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Doping control analysis of small peptide hormones

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List of abbreviations

α-CT	alpha chymotrypsin
AA	amino acid
AAF	adverse analytical finding
AAS	anabolic androgenic steroid
ABP	athlete biological passport
ACN	acetonitrile
ACTH	adrenocorticotropic hormone
ADH	antidiuretic hormone
AGC	automatic gain control
AMPK	AMP-activated protein kinase
AVP	arginine vasopressin
CE	collision energy
CID	collision-induced dissociation
Da	dalton
DoCoLab	Doping Control Laboratory – Ghent University
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
ESI	electrospray ionization
eV	electron volt
FA	formic acid
FSH	follicle-stimulating hormone
FSMS	full scan mass spectrometry
GC-MS	gas chromatography-mass spectrometry
GH	growth hormone

GHRH	growth hormone-releasing hormone
GHRP	growth hormone-releasing peptide
GMP	Good manufacturing practice
GnRH	gonadotropin-releasing hormone
GRF	growth releasing factor
HCD	high-collision energy dissociation (trademark from Thermo Scientific)
HESI	heated electrospray ionization
hGH	human growth hormone
HLB	hydrophilic/lipophilic balance
HLM	human liver microsomes
HOAc	acetic acid
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
IAAF	International Association of Athletics Federations
IAP	immunoaffinity purification
IEF	isoelectric focusing
IGF-1	insulin-like growth factor 1
IOC	International Olympic Committee
ISL	International Standards for Laboratories
ISO	International Organization for Standardization
ISTD	internal standard
kDa	kilodalton
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LOD	limit of detection
m/z	mass-to-charge ratio

MCX	mixed-mode cation exchange (trademark from Oasis)
MeOH	methanol
MGF	mechano growth factor
MRM	multiple reaction monitoring
MRPL	minimum required performance level
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
NADPH	nicotinamide adenine dinucleotide phosphate
NCE	normalized collision energy
PK	pharmacokinetics
PPARδ	Peroxisome proliferator-activated receptor delta
Q-TOF	quadrupole-time of flight
RSD	relative standard deviation
SD	standard deviation
SDS-PAGE	sodium docecyl sulphate-polyacrilamide gel electrophoresis
S/N	signal to noise ratio
SPE	solid phase extraction
SPPS	solid phase peptide synthesis
TA	Testing Authority
Tβ4	thymosin beta 4
TFA	trifluoroacetic acid
tMSMS	targeted tandem mass spectrometry
tR	retention time
tSIM	targeted single ion monitoring
TSQ	triple stage quadrupole
UCI	Union Cycliste Internationale
UHPLC	ultrahigh-performance liquid chromatography

WADA	World Anti-Doping Agency
WCX	weak cation exchange (trademark from Oasis)
X-CW	weak cation exchange (trademark from Phenomenex)

Amino acid abbreviation table

Amino Acid	Three-Letter Abbreviation	One-Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Part I - Introduction

Chapter 1

General introduction

1 Doping and sport

1.1 Sport, doping and society

Throughout history, sports have played a major role in many aspects of human societies. From ancient Greece to the modern times, sport has often been used as a means to influence diplomatic, social, and political relations between countries, to promote political ideals through propaganda, or to express national unity[1,2].

This close relationship between sport and society is explained in good part by the fact that humans have an innate sense of competition. Sport is able to suffice this need by providing an artificial competition (against their own limits, against other humans, or against nature), which is codified by rules that are in accordance with positive values and ethical principles such as fair play, friendship, team spirit, and hard work to obtain achievements[3]. In the words (that became the Olympic creed) of Pierre de Coubertin[4], father of the modern Olympic Games, the spirit of sport is summarized:

"The important thing in the Olympic Games is not to win, but to take part; the important thing in Life is not triumph, but the struggle; the essential thing is not to have conquered but to have fought well. To spread these principles is to build up a strong and more valiant and, above all, more scrupulous and more generous humanity."

Doping is one of the most serious threats to these principles and in general to sport ethics, being a practice that affects the image of sport besides posing a serious risk to individual health. The use of performance enhancing drugs is in fact an illegal shortcut athletes use to reach success in sport, that provide to an athlete an unfair advantage relative to a "clean" competitor[5].

1.2 Brief history of modern doping and anti-doping

Since the antiquity, the use of performance-enhancing agents by athletes (but also soldiers and gladiators) is documented. These substances consisted of preparations made from natural sources, such as herbs and fungi, containing for example caffeine, or strychnine[6].

The first documented case of doping in the modern Olympic Games involves Thomas Hicks, the winner of the Helsinki 1904 marathon competition, who reportedly used strychnine and brandy during the race[7].

Advances in pharmaceutical research and production in the 20th century led to the development of synthetic chemicals and hormones. These drugs were designed with specific therapeutic indications; nevertheless, several of them were soon recognized by the athletes as a powerful way to boost their performances.

Starting from the 1930s, amphetamines began to replace “natural” stimulants such as strychnine. These drugs were used to suppress feelings of fatigue, mainly in endurance sports such as cyclism. Stimulants have been used for years regardless of the health-related risk. The deaths of the Danish cyclist Knud Enemark Jensen at the Rome Olympic Games (1960) and of the British Tom Simpson at the Tour de France (1967) were linked to rumored stimulant abuse, though not confirmed[6,7].

In 1967, the International Olympic Committee (IOC), triggered by these alarming events, instituted a Medical Commission to set up its first (short) list of prohibited substances. Over the years, new doping substances became available, and therefore the list of prohibited compounds was continuously updated[8,9].

In the following years, other federations such as International Association of Athletic Federations (IAAF) and International Cycling Union (UCI) followed IOC with establishing their own anti-doping rules and introducing drug testing procedures. In 1968, drug tests were performed for the first time at the Winter Olympic Games in Grenoble and at the Summer Olympic Games in Mexico[9].

In the 1950s and 1960s, following amphetamines, the use of anabolic steroids became widespread. In the 1952 Olympics in Helsinki, the weightlifters from Soviet Olympic team first used testosterone to increase strength and power. In the 1960s, East Germany started a systematic doping program of performance enhancement to their athletes that would last until the end of the Cold War and the reunification of Germany. These treatments led to numerous gold medals, but also to severe physical and psychological changes in the athletes, due to the consequences of androgen administration[10,11].

Reliable test methods for the detection of anabolic androgenic steroids (AAS) were introduced in 1974. Meanwhile, new synthetic analogues of testosterone (synthetic steroids) became available on the market and were at the same time very attractive to athletes to improve their performances. Due to the large misuse of these substances, the IOC added AASs to its list of prohibited substances in 1967[12].

In the beginning of the 1980s, sport drug testing made an important step forward with the introduction of gas chromatography coupled to mass spectrometry, which provided more effective analyses[13]. The first GC-MS screening methods were successfully implemented at the 1983 Pan American Games and then in 1984 Olympic Games in Los Angeles. One of the most famous cases of doping occurred in 1988 Olympic Games in Seoul, when the detection of stanozolol by GC-MS led to the disqualification of sprinter Ben Johnson and the loss of his gold medal[14]. Nowadays, GC-MS is still the primary method of choice for AASs and together with liquid chromatography-mass spectrometry (LC-MS) represents the most used standard techniques in doping analysis[15].

Besides stimulants and AAS, other drugs found their way into sport and therefore were prohibited[16]. Narcotics can be used to reduce pain in particular sport (e.g.: combat sports) and have been prohibited since 1967. Glucocorticoids, which allow better performance in endurance sports competitions suppressing pain and inflammation, were banned in 2004. Cannabinoids (natural and synthetic) are prohibited since 1989 for their relaxing effect that can produce an indirect advantage to the athlete, and the risks associated to their use in some sports (e.g.: driving sports), and their use is regarded as being in contradiction with the spirit and the image of sport. Beta-blockers, able to reduce heart rate and thus blood pressure, were suspected to be used in those sport were calmness and control of the movements are required (e. g.: archery, shooting sports)[16].

Diuretics, on the other hand, were not used to enhance performance but were considered “masking agents” that could be used to dilute the presence of illegal substances and aid their excretion, impairing their detection. They can also be used to excrete water for rapid weight loss (relevant for some sport with weight categories such as boxing)[17]. Both beta-blockers and diuretics were banned in 1985. Since 1993, beta-2 agonists are prohibited as well because of their stimulating and/or anabolic effects[18].

A more recent class of doping substances is represented by hormone antagonists and modulators, mainly with antiestrogenic activity, which increase indirectly testosterone production by altering biosynthetic pathways and hormone-receptor interactions. Metabolic modulators such as peroxisome proliferator-activated receptor delta (PPARδ) agonists (e.g. GW-1516), AMP-activated protein kinase (AMPK) axis agonists (e.g. AICAR) are also prohibited[19].

All the classes mentioned so far consist of small molecule drugs which are currently monitored by anti-doping laboratories by using multi-analyte GC-MS and LC-MS methods, capable to screen simultaneously for hundreds of compounds from different classes[15,20–22]. These methods have become increasingly effective over the years, taking advantage of new available technologies and progress in research[23–25]. Other substances and methods have emerged during the years which required new approaches and development of new, dedicated methodologies[26].

Peptide hormones made their appearance in the 1980s, taking advantage of newly introduced recombinant technologies, and were prohibited in 1989[27]. Recombinant erythropoietin (EPO), an erythropoiesis-stimulating agent, and growth hormone (hGH) were already available in the 1980s and are still among the most abused doping agents. Detection of their misuse represents a major front in the anti-doping war[6,10,28]. In recent years, the list of prohibited peptide hormones has grown exponentially. Peptide hormones, particularly “small” peptides (molecular weight < 2 kDa) represent one of the main objects of this thesis[29].

Blood doping, defined as "the administration of blood or blood-related products in order to increase the number of red blood cells in the body”, started to be used in the 1970s. Except for anecdotal evidences, the first known case of blood doping was in fact recorded in 1980 Summer Olympics in Moscow in the 5 and 10 kilometer track races. Five years later, blood doping was officially banned[30,31]. An historical view of the use of doping substances is provided in Figure 1.1

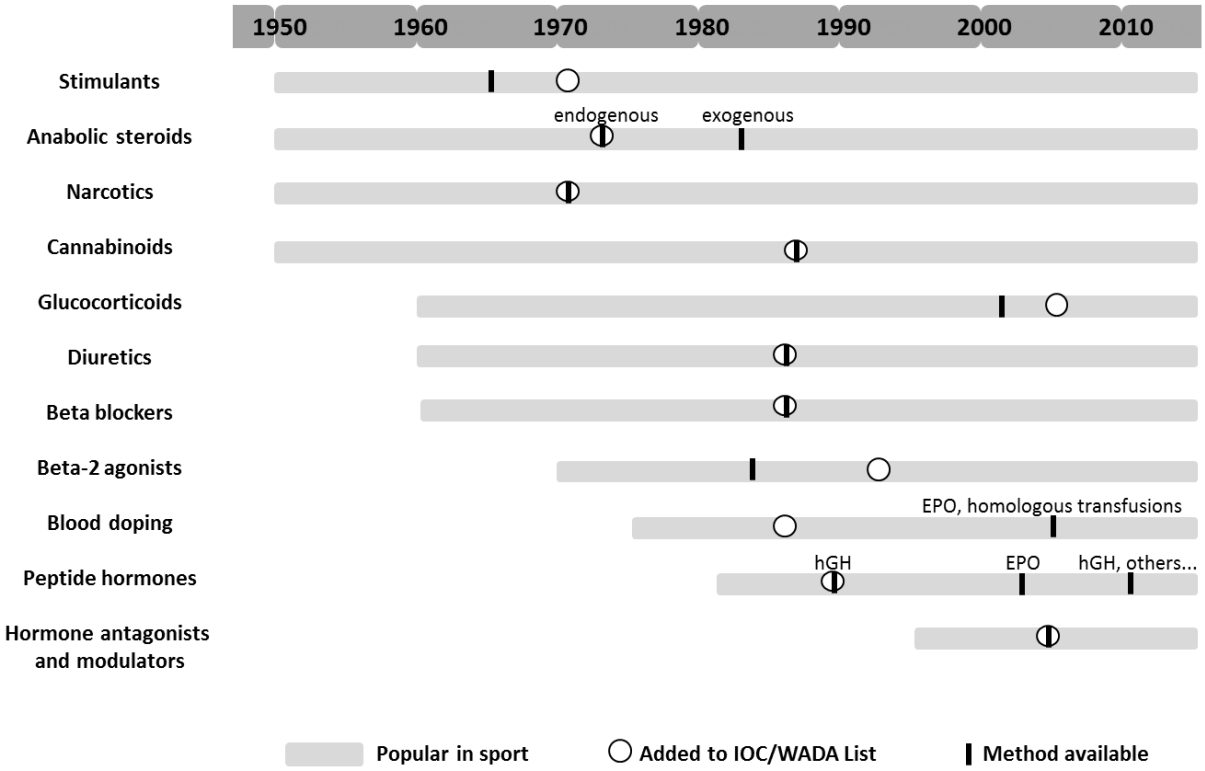


Figure 1.1 Historical view of the (estimated) use of doping substances and methods over the decades, their prohibition from IOC/WADA and the implementation of detection methods.

As the complexity of the doping phenomenon grew significantly during the decades (also as a matter of public health), it became necessary to obtain harmonization and standardization on the fight against doping in sport.

After a major doping scandal during the 1998 Tour de France, a World Conference on Doping was organized in 1999 with the participation of the IOC, Governments, Federations and Institutions. The main result of this conference was the establishment of the World Anti-Doping Agency (WADA), an independent organization with the aim of coordinating the fight against doping[32].

2 World Anti-Doping Agency (WADA)

Since its establishment, WADA has had the purpose to ensure a harmonized approach to anti-doping in all sports and all countries through developments of protocols and guidelines, collaboration with Law Enforcement, coordination of anti-doping activities, and promoting research, doping prevention and education.

One of the most significant achievements has been the implementation of a harmonized set of anti-doping rules, the World Anti-Doping Code (Code)[33,34].

As stated in the Code, the purposes of the World Anti-Doping Code and the World Anti-Doping Program which supports it are:

- *To protect the Athletes' fundamental right to participate in doping-free sport and thus promote health, fairness and equality for Athletes worldwide, and*
- *To ensure harmonized, coordinated and effective anti-doping programs at the international and national level with regard to detection, deterrence and prevention of doping.*

Furthermore, WADA published since 2004 the List of Prohibited Substances and Methods (List), which, as the name suggests, includes all the drugs and the doping procedures which are not allowed in sport (Table 1.1)[35].

A substance or a method can be included in the List in case two of the following conditions are fulfilled:

- *Medical or other scientific evidence, pharmacological effect or experience that the substance or method, alone or in combination with other substances or methods, has the potential to enhance or enhances sport performance;*
- *To protect the Athletes' fundamental right to participate in doping-free sport and thus promote health, fairness and equality for Athletes worldwide, and*
- *Medical or other scientific evidence, pharmacological effect or experience that the use of the substance or method represents an actual or potential health risk to the Athlete;*
- *WADA's determination that the Use of the substance or method violates the spirit of sport described in the Introduction to the Code.*

Since 2004, WADA is responsible for accrediting Anti-Doping laboratories that apply analytical methods and processes to provide evidentiary data for the monitoring of prohibited substances and methods[36].

Table 1.1 Categories of prohibited substances and methods in the 2014 List

Prohibited at all times	
S0	Non-approved substances
S1	Anabolic agents
S2	Peptide hormones, growth factors and related substances
S3	Beta-2 agonists
S4	Hormone and metabolic modulators
S5	Diuretics and other masking agents
M1	Enhancement of oxygen transfer
M2	Chemical and physical manipulation
M3	Gene doping
Prohibited in competition	
S6	Stimulants
S7	Narcotics
S8	Cannabinoids
S9	Glucocorticosteroids
Prohibited in particular sports	
P1	Alcohol
P2	Beta blockers

3 WADA accredited laboratories

Currently, there are 32 WADA accredited laboratories around the world[37]. These structures have the essential role of performing doping control analyses. Furthermore, the laboratories are also carrying research programs of utmost importance for the constant improvement of drug testing methods. Doping control tests can be performed only by WADA accredited

laboratories. In order to achieve and maintain WADA accreditation, a laboratory must fulfill the criteria specified by the International Standard for Laboratories (ISL) and its related Technical Documents[36].

3.1 Analytical strategies in doping control laboratories

Doping control analyses are generally performed in urine, a matrix that provides a prolonged detection time window, and less often in other matrices (blood, plasma, serum, hair…).

The analysis of a doping sample begins with an initial testing procedure (screening). A screening analysis consists generally of a multi-analyte method that is used to detect suspicious or abnormal samples for more detailed analysis[36].

A good screening method must:

- be able to identify a high number of different substances, or different classes in a single analysis (cost effectiveness)
- guarantee detection of any compound at half the concentration of the established minimum required performance limit (MRPL)
- have a high sample throughput and robustness
- not generate any false negative results
- minimize the number of false positive results

Numerous works have been published during the years on GC-MS and LC-MS screening analysis. The trend described by these publications shows how laboratories have moved from multi-analyte, single-class[38,39] to multi-analyte, multi-class methods[23,24]. However, some substances or methods (e.g.: hGH, blood doping…) still require dedicated procedures.

Once a suspicious sample has been identified, a confirmatory analysis is requested. With few exceptions, confirmation methods are also mainly based on GC-MS and LC-MS techniques, as also recommended by the WADA International Standard for Laboratories[36].

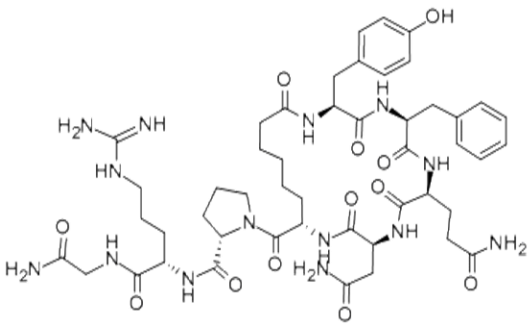
The use of the prohibited compound is confirmed when the analytical data fulfills the criteria given by WADA in comparison to the positive control sample. In this case, the laboratory must report the Adverse Analytical Finding (AAF). In the case of an AAF, the laboratory will inform the testing authority (TA). The TA will connect the result with the athlete on the basis of the sample code[36].

In the last years, a major step forward in the fight against doping has been made with the introduction of the Athlete Biological Passport (ABP). The ABP is generated by measurements of different biological parameters that are influenced by use of doping agents through the time and for each athlete[40].

Currently, the ABP consists of the 'Hematological Module', introduced in 2009 for the detection of blood doping[41], and Steroidal Module, approved since January 2014, to detect steroid doping[42].

4 Peptide hormones and doping

Peptides and proteins consist of chains of amino acids (AA) linked through amide (peptidic) bond. An arbitrary distinction based on the number of AA is made between peptides (2-50 AA) and proteins (> 50 AA) (Figure 1.2).



Arginine vasopressin
(Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2)

Human growth hormone, 22 kDa isoform
(191 aa)

Figure 1.2 Examples of a short peptide (arginine vasopressin – source: http://www.chemicalbook.com/) and a bigger protein (human growth hormone – source: http://en.wikipedia.org/)

Peptide hormones are biochemical compounds synthesized by glands and then secreted to control body physiology and behavior. Together with monoamines (e.g. adrenaline) and

lipid-derived hormones (e.g. steroids), peptide hormones are the three main chemical classes of hormones in human and, in general, in vertebrates[43].

Similarly to what happened to monoamines (stimulants) and lipid hormones (AAS), analogues of peptides have been developed by the pharmaceutical industry to mimic naturally occurring protein hormones for therapeutic purposes, particularly when recombinant technologies became available (Figure 1.3). In 2013, there were around 60-70 approved peptide drugs in the global market, with 100-200 more in clinical trials, 400-600 more in pre-clinical studies[44]. Likewise, an increasing number of peptide hormones has entered in the doping arena.

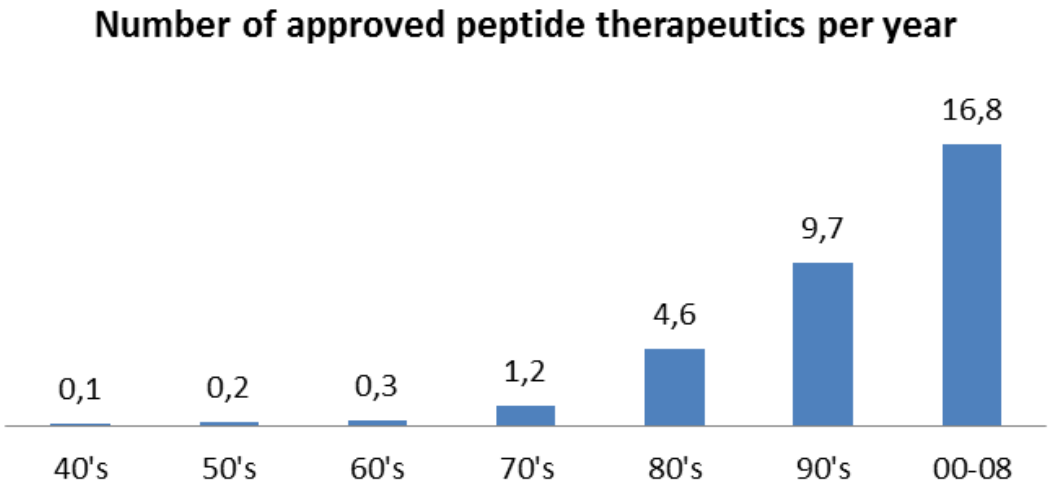


Figure 1.3 Number of approved peptidic drugs per year over the decades.

4.1 Classification by WADA

Peptide hormones are classified in the 2015 WADA Prohibited List under section S2[35]:

"S2. PEPTIDE HORMONES, GROWTH FACTORS, RELATED SUBSTANCES AND MIMETICS"

The following substances, and other substances with similar chemical structure or similar biological effect(s), are prohibited:

1. Erythropoietin-Receptor agonists:

> *1.1 Erythropoiesis-Stimulating Agents (ESAs) including e.g. darbepoietin (dEPO); erythropoietins (EPO); EPO-Fc; EPO-mimetic peptides (EMP), e.g. CNTO 530 and peginesatide; and methoxy polyethylene glycol-epoetin beta (CERA);*
>
> *1.2 Non-erythropoietic EPO-Receptor agonists, e.g. ARA-290, asialo EPO and carbamylated EPO;*

2. Hypoxia-inducible factor (HIF) stabilizers, e.g. cobalt and FG-4592; and HIF activators, e.g. argon, xenon;

3. Chorionic Gonadotrophin (CG) and Luteinizing Hormone (LH) and their releasing factors, e.g. buserelin, gonadorelin and triptorelin, in males;

4. Corticotrophins and their releasing factors, e.g corticorelin;

5. Growth Hormone (GH) and its releasing factors including Growth Hormone Releasing Hormone (GHRH) and its analogues, e.g. CJC-1295, sermorelin and tesamorelin; Growth Hormone Secretagogues (GHS), e.g. ghrelin

and ghrelin mimetics, e.g. anamorelin and ipamorelin; and GH-Releasing Peptides (GHRPs), e.g. alexamorelin, GHRP-6, hexarelin and pralmorelin (GHRP-2).

Additional prohibited growth factors:

Fibroblast Growth Factors (FGFs); Hepatocyte Growth Factor (HGF); Insulin-like Growth Factor-1 (IGF-1) and its analogues; Mechano Growth Factors (MGFs); Platelet-Derived Growth Factor (PDGF); Vascular-Endothelial Growth Factor (VEGF) and any other growth factor affecting muscle, tendon or ligament protein synthesis/degradation, vascularisation, energy utilization, regenerative capacity or fibre type switching.

Regardless of the pharmacological/doping effect, the use of an unapproved substance (including peptide hormones) is prohibited according to the section S0:

S0. NON-APPROVED SUBSTANCES

Any pharmacological substance which is not addressed by any of the subsequent sections of the List and with no current approval by any governmental regulatory health authority for human therapeutic use (e.g.: drugs under pre-clinical or clinical development or discontinued, designer drugs, substances approved only for veterinary use) is prohibited at all times.

As extensively discussed further in this study, the misuse of non-approved formulations containing peptide hormones has represented an emerging and already relevant phenomenon in the recent years.

Furthermore, the peptide desmopressin is banned as an example of masking agent for blood doping (Section *S5. DIURETICS AND OTHER MASKING AGENTS*).

4.2 Doping control analysis of peptide hormones: "size-dependent" approaches

Detection of peptide hormones is currently one of the most challenging fronts in the fight against doping[26,27,45]. As evident in the definition present in the WADA List, the term "peptide hormones" includes numerous compounds with high diversity in terms of size and pharmacological properties.

As a consequence of this variety, it is difficult to analyze simultaneously different substances or different classes of substances, unlike for small molecules. Therefore, whereas for classic, small molecules multi-analyte screening methods are well established and allow screening simultaneously for hundreds of compounds, analytical methods for peptides consist essentially of single- or oligoanalyte methods[46,47]. In this latter case, all the compounds usually belong to the same class.

Besides the limitations related to the large array of prohibited peptide hormones, several other factors concur to make peptide hormone testing a complicated task:

- Elevated similarity of some of the substances (e.g. EPO, hGH) to their endogenous analogues (more difficult to find a marker of misuse); indirect methods (ABP) may be necessary
- Short half-life/detection window compared to non-peptidic drugs (hours vs. days)
- Relatively high chemical instability, particularly if methionine (oxidation), asparagine or glutamine (deamidation) are present
- Low plasma/urine concentrations (nano-, femtomole range)
- More critical sample preparation than for small molecules: non-specific adsorption on surfaces, variable recovery, loss of analyte during transfer and drying steps can cause poor or irreproducible results
- Unfavorable mass spectrometry behavior (mass spectrometry is the most used detection technique in sport drug testing: MS signal generally decreases with the mass of the intact protein)[48]
- Lack of reference standards material

However, a continuous, big effort has been put by anti-doping laboratories in the development of methods over the last decades, resulting in the development of sensitive and reliable methods for detection of peptide hormones. The development and continuous improvements of the methods for EPO and hGH[49] are a clear example. Despite representing

only a minor percentage (< 5%) of the total, the number of AAF related to misuse of peptide hormones is following a growing trend[50], also due to the improvements achieved in detection techniques (Figure 1.4).

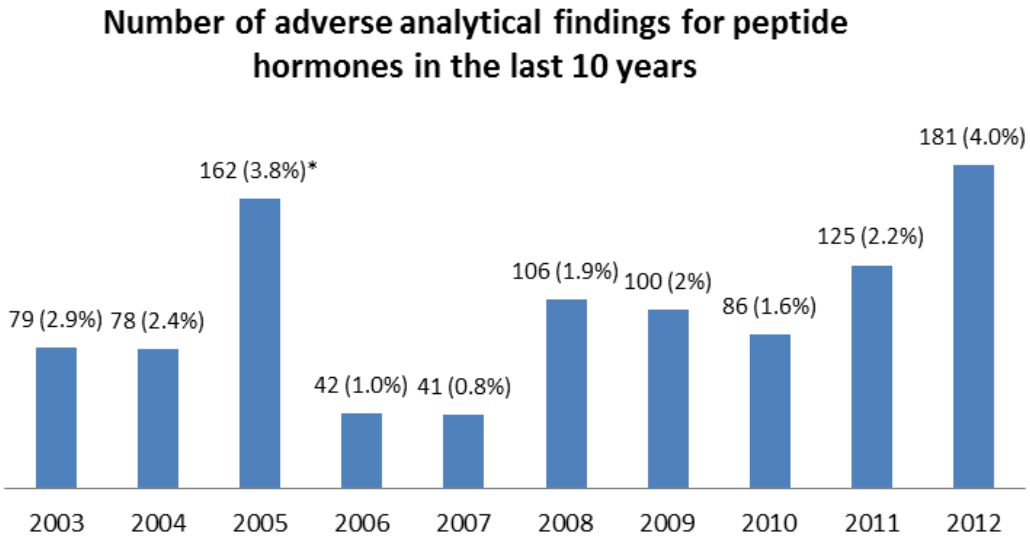


Figure 1.4 Number of AAF related to peptide misuse. (*)2005 statistics included also hCG AAFs in women.

In recent years, numerous other peptide hormones have reached the focus of being misused for their performance-enhancing properties. Similarly, several detection methods have been published. These analytical methods can be roughly divided into two categories:

- MS-based methods[51,52]
- Immunoassays[53–55]

As already mentioned, mass spectrometry is the preferred technique in doping control analysis. Compared to immunoassays such as enzyme-linked immunosorbent assay (ELISA), mass spectrometry provides more adequate selectivity for sport drug testing, being much less likely affected by cross-reactivity with metabolites and other substances other than the target analyte. Over the last years mass spectrometry has emerged as a valid tool for the analysis of peptides also in other fields, particular in clinical analysis for discovery of biomarkers[20,56–58].

Several works concerning LC-MS detection/determination of prohibited peptide hormones have been published in the last decade. These methods can be grouped according to the

sample preparation strategy: solid-phase extraction (SPE)[51] and immunoaffinity purification (IAP)[52].

Among the advantage of using SPE:

- Cost effectiveness: SPE cartridges are much less expensive than antibodies and related tools (e.g. immunomagnetic beads or immunoaffinity columns)
- Possibility to analyze numerous peptides from different classes: selectivity of purification does not depend on antigen-antibody interaction
- generally easily expandable to detect new emerging peptides

Among those of using IAP:

- High specificity for the analyte, which reflects in extremely clean extracts, better quality of the MS signal (virtually no ion suppression) and provides confidence in the analytical results
- Gentle conditions: organic solvents or strong acids/bases are not used
- Time effective: no evaporation step needed

As already mentioned, detectability of peptides becomes more difficult with increasing size. For peptides with a molecular weight > 2-3 kDa, almost all analytical methods described in literature involve the use of IAP. Due to the higher complexity and instability of the molecule and the general mass-dependent loss of MS sensitivity, the high purity of the injected samples and the gentle sample handling become utmost important to obtain sensitive and reliable methods.

However, for bigger proteins such as hGH or EPO-related substances, mass spectrometry currently seems to lack sensitivity to detect the presence of the substance or provide a quantification. For example, hGH doping can currently be detected by the so-called “isoform differential immunoassay” and through determination of indirect serum markers[55]. Detection of EPO is based on isoelectric focusing (IEF)[53]. Human chorionic gonadotropin (hCG) misuse is also monitored by immunoassays[54]. Methods based on mass spectrometric detection of IGF-1, EPOs and hCG have been developed with promising results but their suitability for doping control still has to be determined[46,56]. Figure 1.5 summarizes this classification.

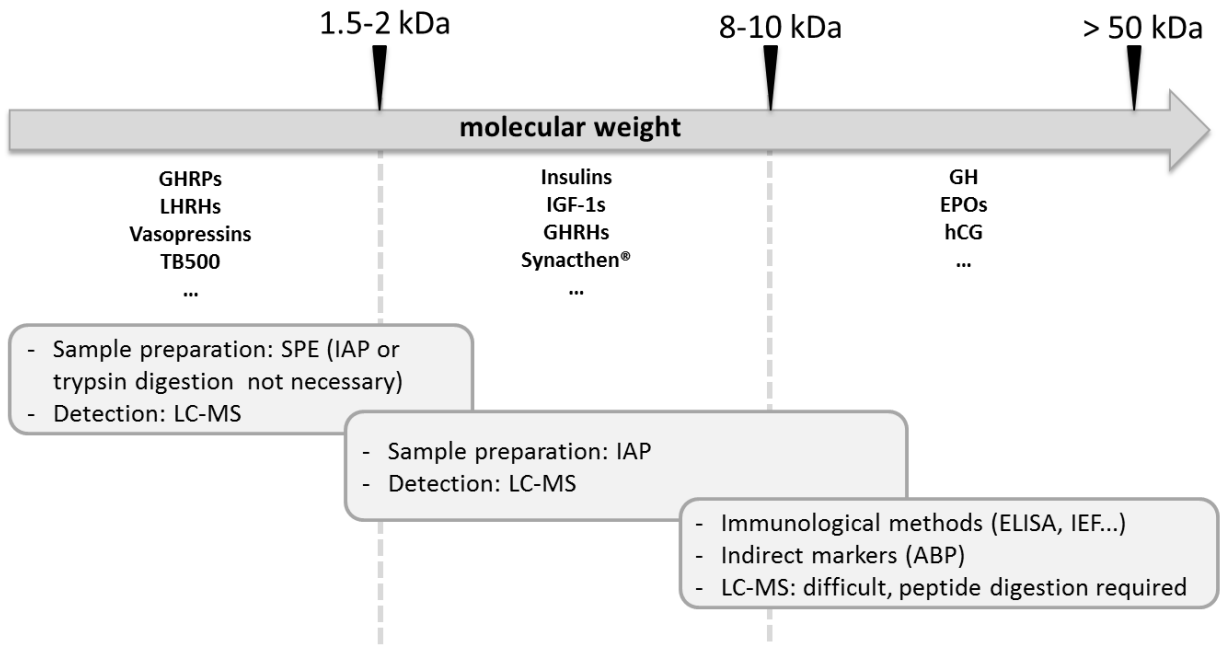


Figure 1.5 Scheme of the size-dependent classification of peptide hormones and the analytical techniques used for their detection.

4.3 Small peptide hormones

There are several major categories of prohibited peptide hormones and the compounds vary greatly in size, effects, and mechanism of action. This study focused mainly on the development of analytical methods for the detection of small (< 1.5-2 kDa) peptides, though in the context of black market of peptide hormones (see Part III), peptides of bigger size are described.

Overall, the peptides investigated in this study can be grouped in four classes, based on their mode of action and pharmacological/doping/masking effect:

1) *Luteinizing hormone-releasing hormone (LHRH) agonists*

   *S*ynthetic derivatives of the decapeptide LHRH, designed to improve the pharmacokinetic properties of the endogenous peptide, activate the LHRH receptor resulting in increased secretion of follicle-stimulating hormone (FSH) and luteinzing hormone (LH). This class of drugs was originally developed for treatment of several conditions, including prostate cancer, estrogen-related disorders and gender identity related disorders. However, since intermittent application of GnRH agonists increases plasma testosterone concentrations, these drugs have gained relevance also in doping controls[59–61].

2) *Growth-promoting hormones acting on the growth hormone/insulin-like growth factor 1 (GH/IGF-1) axis*

IGF-1 is a potent anabolic hormone, mainly secreted by the liver after stimulation by GH. Synthesis and release of GH from the pituitary gland is regulated positively by the growth hormone-releasing hormone (GHRH) and negatively by somatostatin, both secreted by the hypothalamus. GH/IGF-1 axis-related peptidic drugs are generally used for diagnosis and treatment of GH deficiency and related disorders[62].

It is easy to imagine how this class of compounds can be attractive to cheating athletes. In fact, numerous peptidic doping agents acting at different points of the GH/IGF-1 axis have been described[63]. Limiting the list to those included in this study (and thus not including many others such as recombinant GH and IGF-1), the following sub-classes are described:

- Growth hormone-releasing hormone (GHRH) analogues: synthetic derivatives of the hypothalamic 44-AA hormone that similarly increase GH production[64,65]
- Growth hormone-releasing peptides (GHRPs): synthetic, non-natural oligopeptides that stimulate GH secretion but have no homology with GHRH[64,65]
- Mechano-growth factors (MGFs): analogues of human MGF, a 28-AA, splice variant of IGF-1 produced in the liver that induce muscle growth and hypertrophy in case of mechanical stimulation or damage[66]
- IGF-1 and analogues
- GH fragments, particularly fragment 176-191 of GH, a portion of the molecule with lipolytic effects[67]

3) *Arginine vasopressin (AVP) analogues*

AVP, also known as antidiuretic hormone (ADH), is a cyclic nonapeptide that regulates body water retention by increasing water reabsorption in the kidney nephron[62]. AVP analogues, particularly its long-acting synthetic analogue desmopressin are used mainly for treatment of nocturnal enuresis, coagulation disorders and diabetes insipidus[68]. Due to the fact that AVP analogues can cause hemodilution, they can act as masking agents by altering the values of hematological parameters used to detect blood doping in the framework of the ABP[69]

4) *Thymosin beta-4 (Tβ4)fragments*

The fragment 17-23, responsible for the actin-sequestring activity of the 43-AA peptide Tβ4[70], is currently not approved for any therapeutic application and it is illegally traded under the name TB-500. Due to its potential role in muscular and connective tissue regeneration, an use for similar purposes to those of MGFs can be hypothesized.

As in the case of TB-500, some of these peptides are not approved for therapeutic use. The main source for these compounds is represented by the so-called "black markets".

4.4 Black market of performance enhancing drugs

Most of the substances used for doping are usually designed as treatments for specific diseases[14,16]. Even blood transfusion and blood doping originate from an emergency treatment in the case of critical and copious bleeding. Trafficking of doping products has grown exponentially over the decades parallel to evolution of doping itself (appearance of new doping agents), pharmaceutical research (new therapeutic targets, new synthesis strategies, recombinant technologies) and trading channels (e-commerce)[71].

Black market doping substances can be divided in two categories:

- Approved, controlled substances, used in medical treatments (e.g.: testosterone, hGH, tamoxifen…)
- Unapproved drugs (e.g.: tetrahydrogestrinone, some GHRPs…)

Collaboration between different authorities such as Police, Customs, Health and Anti-Doping Authorities is essential in the fight and prevention of this phenomenon (Figure 1.6). From the perspective of the anti-doping laboratories, drugs from the black market, especially non-approved substances, represent a difficult challenge. The first step consists of characterization of the active content. Once a new substance has been identified, reference standard material is required to set up a new analytical method or, in the best case, include the compound in an already existing screening procedure. Synthesizing reference material can be more or less complicated, depending on the nature of the substance.

Nowadays, peptide hormones represent one of the most popular classes of doping agents available on the black markets, as testified by numerous related cases[72–74], publications[75–77] and the so-called "underground literature" (e.g.: websites, online forums). AAS, selective androgen/estrogen modulators (SARMs and SERMs), beta2-agonists (particularly

clenbuterol), aromatase inhibitors such as anastrazol, and the AMPK activator 5-amino-4-imidazolecarboxyamide ribonucleoside (AICAR) appear to easily find their way in illegal channels of distributions.

Figure 1.6 Example of black market products seized by Belgian Customs and delivered to DoCoLab for identification.

4.5 Peptide metabolism

Peptidic drugs follow the same general pharmacokinetics and metabolism principles of classic, small molecules, but they are metabolized with different enzymes and through different modifications[43].

In fact, small molecules are mainly metabolized in the liver first by introduction of active and polar groups with the help of the cytochrome P450 system (Phase I), then conjugated with charged and polar species such as glucuronic acid to further facilitate drug excretion (Phase II). In some cases, further modifications (e.g. conjugation of glutathione conjugated with acetylcystein) can occur (Phase III)[78].

On the other hand, peptidic drugs undergo aspecific enzymatic proteolysis from peptidases (also called proteases). As a consequence of this, the native peptide is *digested* in smaller peptide fragments or even single amino acids. All peptidases catalyze the hydrolysis of a peptide bond, but they are selective for the position of the substrate and also for the amino acid residues close to the bond that undergoes cleavage[79].

On the basis of the type of cleavage they catalyze, peptidases can be divided in:

- *exopeptidases*, which cleave terminal amino acids from the N-terminus (aminopeptidases) or the C-terminus (carboxypeptidases) of a peptide substrate

- *endopeptidases*, such as trypsin and chymotrypsin, which cleave non-terminal amino acids from a peptide substrate
-

Several peptidases can also have deamidating activity at the C-terminus. These metabolic reaction consists of the hydrolysis of ther amide bond on the last C-term AA. Figure 1.7 summarizes these main degradation pathways.

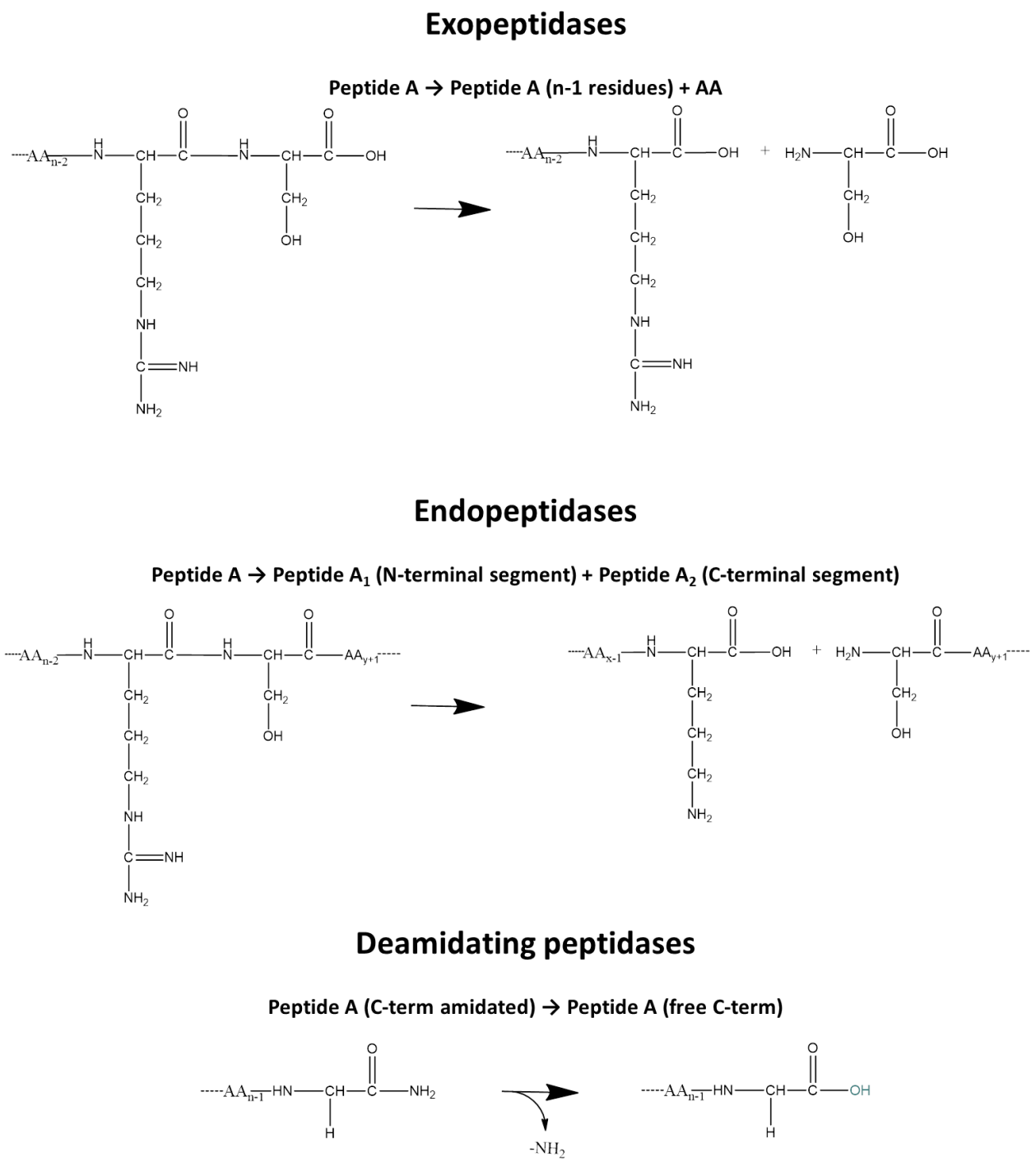


Figure 1.7 Scheme of the major peptide degradation pathways.

Peptide metabolism in not usually limited to the liver: an important (in some cases even primary) role is played also by plasmatic and kidney peptidases.

Peptides with only endogenous AA are generally degraded very fast. Therefore, synthetic derivatives were designed with modification such as:

- substitution of one or more AA
- use of the D enantiomer AA instead of the L
- modification at the terminus (C-term amidation, N-term acetylation)

In doping control analysis, the knowledge of the metabolism of a prohibited substance is of primary relevance, since targeting (long-term) metabolites can prolong the detection time window, thus increasing the possibilities to reveal the abuse of a doping agent. This has been described by numerous publications on different classes such as AAS, diuretics, stimulants and many others[17,39,80,81].

MS-based techniques are currently extensively used in drug discovery and development[82] and drug metabolism research, including not only doping control[83] but also clinical and forensic pharmacology and toxicology[20,84].

First GC-MS and then LC-MS have been established as the premier tool for drug metabolism studies (especially LC-MS for being not limited to the analysis to volatile and thermally stable compounds)[85]. An additional, major improvement was represented by the development of high-resolution mass spectrometry (HRMS) instrumentation, capable to generate molecular weight and structural information on the drug metabolites[86]. Particularly when coupled to high-performance liquid chromatography (HPLC-HRMS), this technique has evolved into an indispensable tool for targeted and untargeted metabolomics analysis. In recent years, HPLC-HRMS has become the core technology also for the qualitative and quantitative analysis of the proteome (proteomics)[87].

Due to the short half-life of most of peptidic-drugs, identification of metabolites can have important applications in doping control analysis. At the time this study was started, only few studies on the metabolism of prohibited peptides were described in literature: some promising results had already been obtained with the identification of insulins[88] and GHRP-2[89] metabolites that resulted in improved detection of misuse of these peptides.

Metabolism studies, particularly for non-approved drugs, require *in vivo/in vitro* models due to the limitations in performing administrations in human. Whereas models for small, non-

peptidic drugs are well established in forensic analysis, with the use of tissue homogenates such as human liver microsomes (HLM)[90] or animal models (including chimeric species)[91], the development of such models for peptidic substances is a more recent issue, as it will also be included in this study.

4.6 Mass spectrometry of peptides

The application of MS for the qualitative and quantitative analysis of peptides and proteins has had a huge impact in a variety of fields, such as molecular and cell biology, drug development, and biomedical and clinical practice. In the last years, the fields of proteomics and peptidomics have emerged with the goals to characterize structurally and functionally respectively proteins and peptides in complex matrices[87,92].

Proteomics is defined as the large-scale study of proteins, particularly their structure and functions[87]. On the other hand, the object of peptidomics is the study of the structure and biologic properties of peptides present in biologic matrices (fluids, tissues, cells...)[58,93].

In both cases, mass spectrometry techniques represent the gold standard, since it allows for accurate determination of the molecular mass of known and unknown proteins/peptides as well as their sequences (*de novo sequencing*), especially after the introduction of HRMS instrumentation. Protein/peptides identification can be performed with two different methods, as shown in Figure 1.8: *top-down* (analysis of the intact peptide) and *bottom-up* (proteolytic digestion of proteins prior to analysis by mass spectrometry of the peptide fragments).

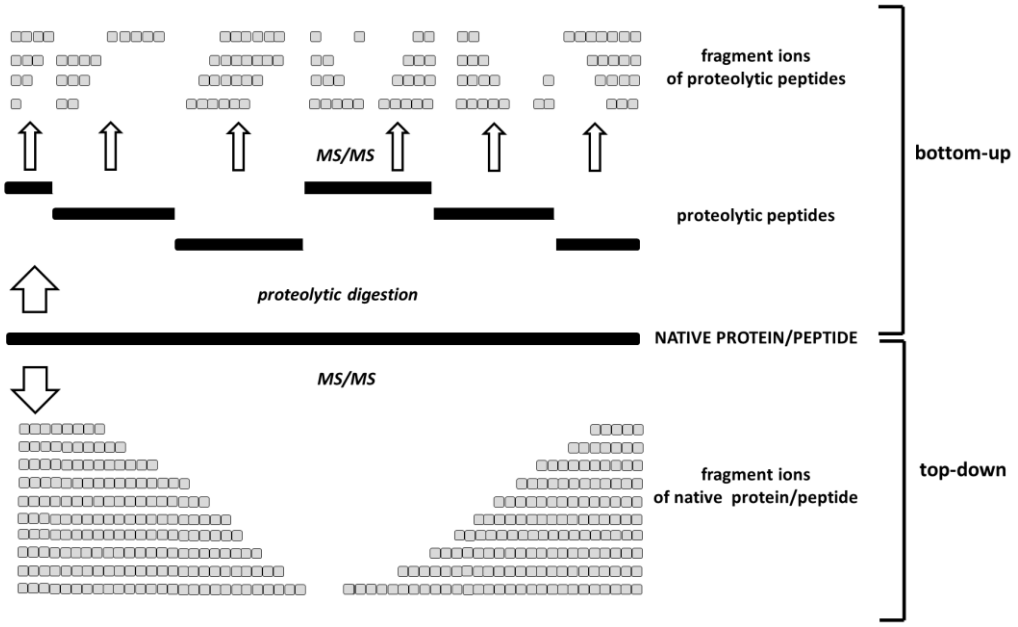


Figure 1.8 *Bottom-up* and *top-down* approaches used in proteomics for protein identification.

Tandem mass spectrometry (MS/MS) experiments are performed both for top-down and bottom-up analyses. In MS/MS experiments, specific precursor ions, corresponding to the intact peptide can be selected to be fragmented. The resulting MS/MS spectra present fragment ion series (Figure 1.9) which can be used to determine the AA sequence[87,92].

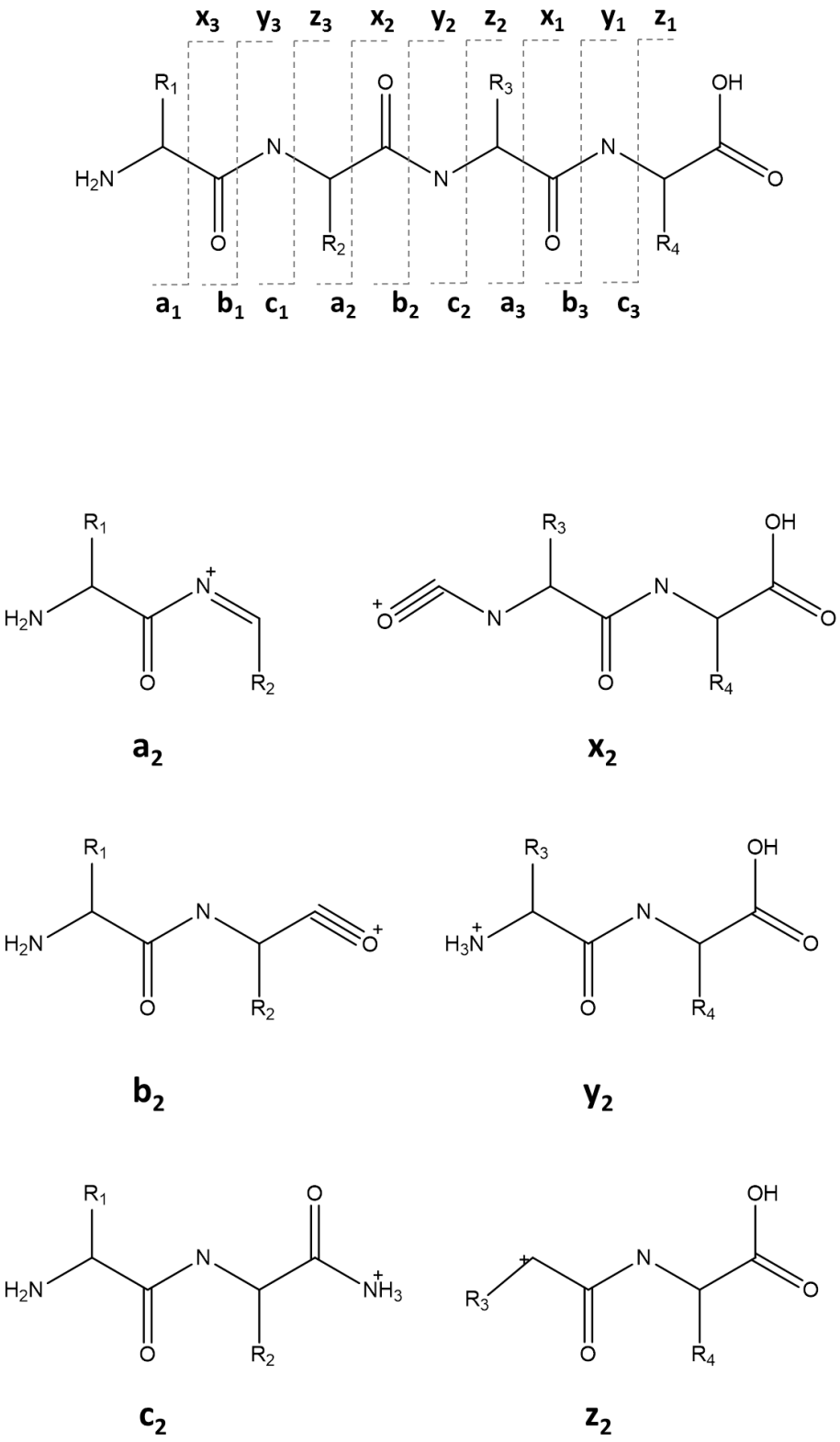


Figure 1.9 Nomenclature of sequence-specific peptide fragments; a-, b-, and c-type ions contain the N-terminus; x-, y-, and z- ions contain the C-terminus. At low collision energy

values, a, b and y ions are the most commons, as also seen in the identification of peptides in Chapters, 6,7and 8. Other characteristic fragments can be formed[92,94]**.**

For the identification of an unknown peptide, additional information can be provided by deconvolution of the spectrum of the intact peptide. This is most commonly done with dedicated softwares, which use algorithms that extrapolate the monoisotopic, single charged peak (and thus the molecular mass) from the *m/z* values of multiple charged peaks and their isotopic clusters.

With the increased popularity of peptide hormones in sport doping, the use of proteomics/peptidomics approaches is clearly acquiring relevance also in the doping arena, including also this thesis, whose purposes will be described in detail in the next chapter.

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Chapter 2

Aim of the study

1 Background and context of the study

Doping control analysis of small peptides can, currently, be considered as a hot topic in sport drug testing. The list of prohibited peptide hormones is constantly evolving: for example, at the time this study was started (January 2011), desmopressin had just been included in the WADA List, whereas some others (e.g.: TB-500) had not been appeared on the scene yet.

At that time, research on small peptides was mainly focused on LHRH and GHRPs. The first LC-MS method for detection of LHRH doping in urine was published in 2008 by Thomas *et al*.[1]. Sample preparation was based on SPE followed by IAP, analogously to previous works on bigger peptides (e.g.: insulins)[2]. In 2010, detection of GHRP-2 and its long-term metabolite (AA 1-3) in urine was achieved by Okano *et al*. using solid-phase extraction for sample pretreatment. In 2011, the first multi- analyte, single-class method for GHRPs was published[3], followed in 2012 by the first multi- analyte, multi-class method[4].

Therefore, from the analytical point of view, the following trends could be observed:

- From SPE+IAP to SPE only for sample preparation
- From single-analyte methods to multi-class methods
- Research on the identification of metabolites and then inclusion in methods to increase detection windows

Simultaneously, several publications started to highlight the phenomenon of black market of peptides hormones with performance enhancing properties, particularly GHRPs and GHRH analogues[5–7].

In such context, this research has been carried at the Ghent University Doping Control Laboratory (DoCoLab), with the overall aim of implementing suitable analytical methods for detection of small peptide hormones. The study has been performed along three main lines of research:

- Development of methods for the qualitative analysis of prohibited peptides in urine and/or plasma
- Development of *in vitro* models to study the metabolism of prohibited peptides

- Identification of unknown peptide hormones with doping potential from black markets

2 Detection of desmopressin in plasma and urine

The first part of the study will focus on the development of new LC-MS/MS methods for detection of desmopressin in human plasma and urine. The AVP analogue desmopressin was added to the WADA List in 2011 as an example of a masking agent[8]. In fact, this peptide can act as a plasma volume expander by decreasing significantly hematocrit and hemoglobin values, and thus masking blood doping.

No LC-MS-based methods for desmopressin plasma had been described before the beginning of the study, but only immunoassays were reported in the literature[9,10]. To fit anti-doping purposes, the method should be able to detect desmopressin unambiguously at the femtomole range and allow for a relatively high throughput (20-25 samples per day).

To achieve this goal, SPE-based sample preparation procedures were evaluated, comparing different SPE stationary phases and different protocols. Detection methods were developed using a high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) system. The MS detector consisted of a low-resolution triple-stage quadrupole mass spectrometer, as this was the only type of MS system available in the laboratory at the time of the desmopressin studies described in Part II of this thesis.

In order to evaluate which matrix is preferred for monitoring desmopressin, detection methods for confirmation and screening in both plasma and urine will be compared.

3 Peptide hormones and black markets

In Part III of this thesis, illegal trading of peptide hormones will be discussed, particularly from the perspective of the anti-doping laboratories.

Over the last years, numerous new products have found their way into black markets[5–7,11]. Identification of these emerging doping substances is the first important step toward detection of their misuse. The existence of a new black market product is usually revealed in two ways:

- Anecdotal evidence: online black markets, internet discussion forums (e.g.: bodybuilding forums)
- Authorities' actions: confiscation of illegal products by Police or Customs.

These two sources are both used by the laboratory to discover new prohibited peptide hormones. Particularly, DoCoLab started collaboration with the Belgian Laboratory of Customs and Excise in Leuven to identify peptide hormones in black market formulations. Several confiscated products by the Customs were sent to the laboratory for identification. Additionally, several products have been purchased directly from online distributors.

For identification purposes, methodologies based upon HRMS coupled to ultra-high-performance liquid chromatography (UHPLC-HRMS), using either bottom-up and top-down approaches will be developed.

The results and characterization of new potential doping agents will be described in Part III.

4 *In vitro* metabolism of small peptides

As first shown by the work of Okano *et al.*[12], monitoring metabolites of small peptides can increase the chances to detect a positive sample. Since performing administration studies of prohibited peptide hormones is not always allowed because of ethical constraints, it is necessary to develop alternative models to elucidate their metabolism, analogously to other classes of doping agents such as designer steroids and synthetic cannabinoids[13,14].

In part IV of this thesis, the use of *in vitro* models to study small peptide metabolism for doping control purposes will be evaluated and compared with recently published literature.

For the evaluation, the results of the following model peptides will be discussed: desmopressin, LHRH, leuprolide, TB-500, GHRP-2, GHRP-6, and hexarelin. Results will be

compared with those obtained with established models (incubation with fresh serum and plasma)[15]. For *in vitro* experiments, the use of human microsomes and fraction S9 from human liver and kidney were tested, despite the fact that these models are conventionally used only for small, non-peptidic drugs. Additionally, deamidation was evaluated by incubation of the peptides with the deamidating enzyme α-chymotrypsin. After incubation, the metabolites were identified by UHPLC-HRMS in MS/MS mode. The results of these experiments will be discussed.

5 Screening method

Over the last years, the number of small peptidic doping agents has increased. With significant efforts from WADA and its laboratories, the knowledge on their detection and on their metabolism has significantly incremented. Main strategies for detection were established; moreover, reference standard and certified reference materials to perform research and to develop analytical methods became more available.

Depending on the fact that a new peptidic dopingagent is approved for therapeutic use or not, there are two major paths that lead from its discovery to its detection (Figure 2.1).. As it will also be discussed in this thesis, an unapproved drug (e.g.: TB-500) yields additional problems to the doping control analyst.

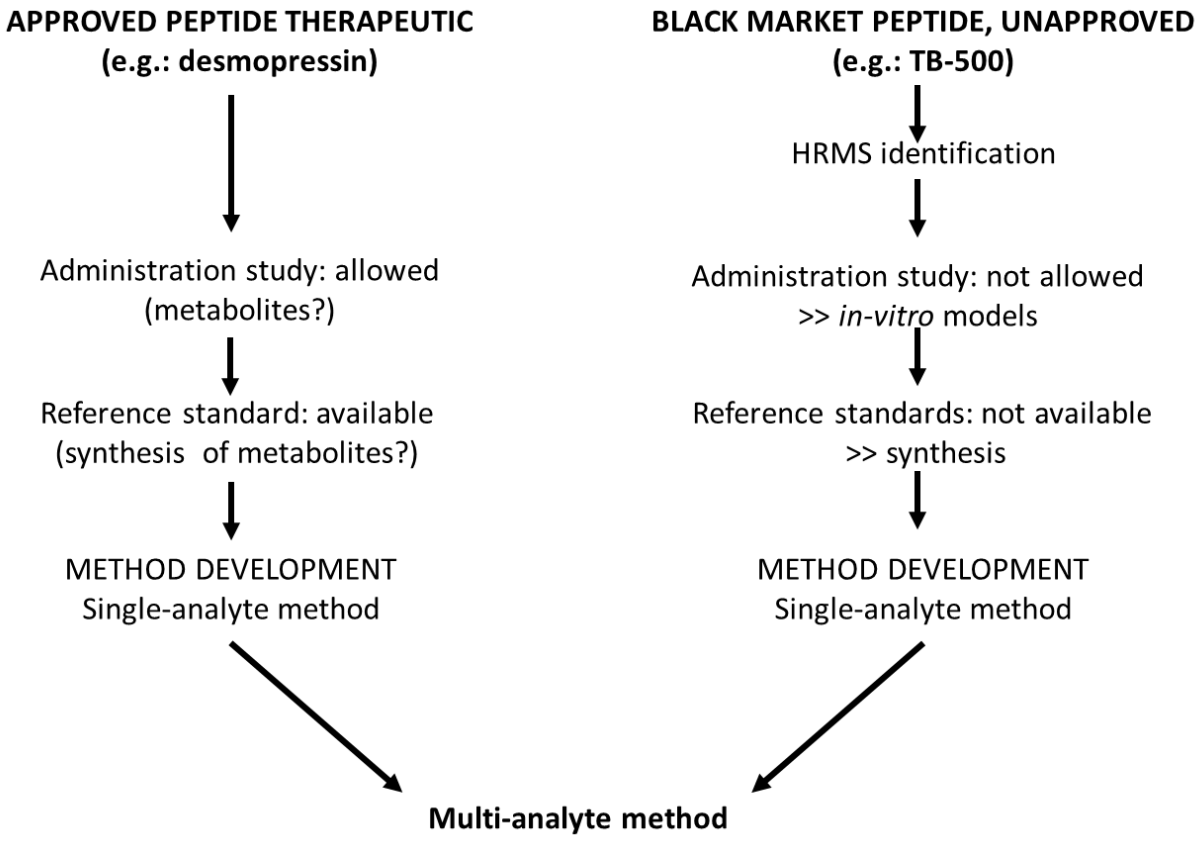

Figure 2.1 Scheme of the paths from discovery to doping control analysis of small peptide hormones.

However, regardless of the status of the peptide, the final goal of this investigation is the implementation of the analysis of small peptide hormones in the laboratory.

As these compounds shared similar analytical issues, a multi-analyte method is preferred in order to increase efficiency of testing and reduce costs.

A UHPLC-HRMS method will be developed and validated in order to detect 31 small peptides, including metabolites:

- Arginine vasopressin analogues: desmopressin, lysine vasopressin, terlipressin
- TB-500 and 3 TB-500 metabolites
- GHRPs: GHRP-1,-2,-4,-5,-6, ipamorelin, alexamorelin, hexarelin, and 5 metabolites (1 for GHRP-2, 2 for GHRP-6, 2 for hexarelin)
- Human LHRH and analogues: buserelin, deslorelin, goserelein, leuprolide, nafarelin, triptorelin, and 3 metabolites (2 for leuprolide, 1 for naftarelin)

- AOD9604

Sample preparation will be based on SPE, similarly to previous works[4,16]. UHPLC-HRMS will be used for the development of the method. All compounds must be detected at sub ng/mL concentration.

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Part II - Detection of desmopressin in plasma and urine

Chapter 3

Detection of desmopressin in plasma by liquid-chromatography-tandem mass spectrometry

Adapted from:

S. Esposito, K. Deventer, G. T'Sjoen, A. Vantilborgh, F. T. Delbeke, A. Goessaert, K. Everaert; P. Van Eenoo. Qualitative detection of desmopressin in plasma by liquid chromatography-tandem mass spectrometry. *Anal. Bioanal. Chem.* ***2012****, 402, 2789-2796.*

1 Abstract

This Chapter describes the development of a liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) method for detection of desmopressin in human plasma.

Desmopressin is a synthetic analogue of the antidiuretic hormone arginine vasopressin and it might be used by athletes as masking agent in the frame of blood passport controls. Therefore, it was recently added by the World Anti-Doping Agency (WADA) to the list of prohibited substances in sport as a masking agent.

Mass spectrometry characterization of desmopressin was performed with a high-resolution Orbitrap-based mass spectrometer. Detection of the peptide in the biological matrix was achieved using a triple quadrupole instrument with an electrospray ionization (ESI) interface after protein precipitation, weak cation solid phase extraction and HPLC separation with an octadecyl reverse phase column. Identification of desmopressin was performed using three product ions, *m/z* 328.0, *m/z* 120.0, and *m/z* 214.0, from the parent ion at *m/z* 535.5.

The extraction efficiency of the method at the limit of detection was estimated at 40% (n=10), the ion suppression at 5% (n=10), and the limit of detection was 50 pg/mL (S/N> 3).

Selectivity of the method was verified against several endogenous and synthetic desmopressin related peptides. The performance and the applicability of the method were tested by analysis of clinical samples after administration of desmopressin via intravenous, oral, and intranasal routes. Only after intravenous administration, desmopressin could be successfully detected.

2 Introduction

Desmopressin, 1-desamino-8-D-arginine-vasopressin (dDAVP), is a synthetic analogue of arginine [Arg8]-vasopressin (AVP), a cyclic peptide hormone with antidiuretic properties, synthesized in the hypothalamic cells and released from neurohypophysis. Desmopressin (monoisotopic mass: 1068.4269 Da) is obtained by deamination at the N-terminal 1 position of AVP and replacement of 8-L-arginine with its D-isomer (Figure 3.1)[1–3].

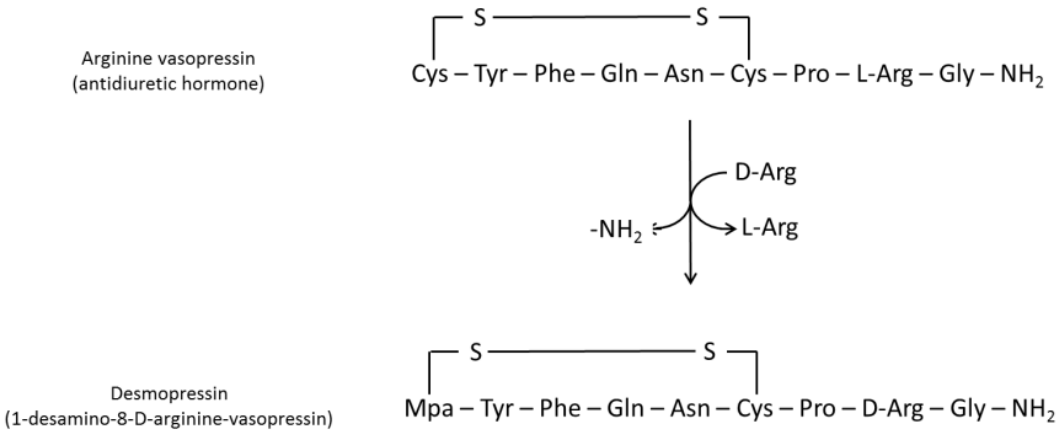


Figure 3.1 Compared to arginine vasopressin, Cys1 in desmopressin has been deaminated, and the Arg8 is in the dextro rather than the levo form.

Compared with AVP, desmopressin has a longer lasting and more potent antidiuretic effect and is devoid of vasopressor effects[2,4,5]. Desmopressin is therapeutically used for the treatment of diabetes insipidus[4,5], primary nocturnal enuresis (bed wetting) [6–10] type I von Willebrand disease, and hemophilia A[11–14].

Desmopressin can induce hemodilution decreasing hematocrit levels and hemoglobin concentrations. These values are important hematological parameters used to detect blood doping in sports[15,16].

Therefore, desmopressin has recently been added by the World Anti-Doping Agency (WADA) to the 2011 Prohibited List as a masking agent[17].

Previous methods for the detection of desmopressin in biological fluids were performed only for clinical purposes, by immunoassays. These methods provided sensitive and cost effective analyses[8,18–22], but with some important limitations, in particular concerning specificity[23].

Liquid chromatography-mass spectrometry (LC-MS) shows good sensitivity together with a high degree of selectivity and specificity to allow for unequivocal confirmation of the presence of a substance based on its molecular weight. Hence LC-MS methods have become the method of choice for the analysis of peptide hormones[24].

Until now, only one study using LC-MS to identify desmopressin has been published[25]. This method was applied for the analysis of skin samples and has a limit of detection (LOD) of 10 ng/mL (≈ 10 picomole). Obviously, this matrix is not applicable in the field of sport drug testing. Hence, the present work had the aim to develop and validate a LC-ESI-MS/MS method for detection of desmopressin in plasma fit for anti-doping purposes.

3 Materials and methods

3.1 Chemicals and reagents

All the chemicals and solvents used for sample pretreatment and chromatography were analytical grade or HPLC grade. Desmopressin was a kind gift from Ferring Pharmaceuticals (Malmö, Sweden). AVP and [deamino-Cys_1, Val_4, D-Arg_8]-vasopressin were purchased from Sigma Aldrich (Saint Louis, USA). Oxytocin, [Lys_8]-vasopressin, and terlipressin were purchased from Selleck Chemicals LLC (Houston, USA). Desmopressin and analogues were all available as acetate salts. Stock and working solution were all prepared using 2% acetic acid (HOAc) aqueous solutions. Acetonitrile (ACN) and water (H_2O) were purchased from BioSolve (Lexington, USA); methanol (MeOH) was purchased from Fisher Scientific (Aalst, Belgium); 25% ammonia (NH_4OH) aqueous solution and glacial HOAc were purchased from Merck (Darmstadt, Germany); trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich.

Saturated ammonium sulfate ($(NH_4)_2SO_4$) solution was prepared by dissolving 800 g of $(NH_4)_2SO_4$ (Merck, Darmstadt, Germany) in 1000 mL of H_2O at room temperature. The

resulting solution was gently poured into a second glass bottle in order to get rid of the undissolved crystals of $(NH_4)_2SO_4$, then 20 mL of 25% NH_4OH were added to 1 l of the $(NH_4)_2SO_4$ solution.

Only low binding microcentrifugal vials, type Eppendorf (Eppendorf, Hamburg, Germany) and low retention pipette tips (Sorenson Biosciences, Salt Lake City, USA) were used to prepare stock solutions (100 µg/mL) and working solutions (10 ng/mL). AVP was always added to desmopressin working solutions at a concentration of 2.5 ng/mL, in order to prevent desmopressin adsorption to solid surfaces.

3.2 Plasma samples

Method development and validation were performed using left-over plasma samples obtained from Red Cross Blood Transfusion Center (Gent, Belgium, approval number 20100609). Desmopressin containing plasma samples were obtained from the Department of Internal Medicine (Endocrinology and Hematology) of the Ghent University hospital, with the approval of the Ethical Committee of the Ghent University (reference: B67020108809).

3.3 Sample preparation

2 mL of plasma were spiked with 50 µl of [deamino-Cys_1, Val_4, D-Arg_8]-vasopressin (50 ng/mL) as internal standard. Then, 2 mL of saturated $(NH_4)_2SO_4$ solution, containing 2% NH_4OH, were added and the samples were centrifuged (4100 g, 1 h, 2°C) to precipitate plasma proteins. A further purification of the supernatant was performed by solid phase extraction with Oasis® WCX (60 mg) cartridges, purchased from Waters (Milford, USA). The column was first activated with 2 mL of MeOH and subsequently rinsed with 2 mL of H_2O. After the sample was loaded, the column was washed first with 2 mL of 5% NH_4OH aqueous solution, then with 2 mL of 60:40 H_2O:MeOH mixture. Finally, samples were eluted with 1.25 mL of a solution consisting of 80:20 MeOH:(5% HOAc in H_2O). The eluate was subsequently evaporated to dryness with a centrifugal evaporator (45°C, 240 g, 6-8 h), and then dissolved in 40 µl of 95:5 H_2O:ACN, 0.1% HOAc, 0.01% TFA prior to LC-MS analysis.

3.4 Liquid chromatography

HPLC separation was achieved with a Surveyor MS Pump Plus coupled with a Surveyor Plus autosampler (Thermo Scientific, Bremen, Germany) using a Zorbax 300SB-C18 reverse-phase

column (1.0 x 50 mm, 3.5 µm) protected with a Zorbax 300SB-C8 guard column, both from Agilent Technologies (Santa Clara, USA). The use of the C8 guard column was adopted from a previous work from Thevis *et al.*[26].

For each sample, 30 µl were injected. A binary gradient was used: mobile phase A consisted of H_2O, 0.1% HOAc, 0.01% TFA; mobile phase B consisted of ACN, 0.1% HOAc, 0.01% TFA. Gradient elution was as follows: 95% A for 1.5 minutes, then decreased linearly to 0% A in 8.5 minutes, and held at 0% A for 5 minutes, followed by an increase to 95% A in 0.1 minutes. Then the system was equilibrated for 10 minutes before next injection (total run time: 25 minutes). A constant flow rate of 50 µl/min was maintained.

3.5 Mass spectrometry

MS/MS method development for detection of desmopressin in human plasma was performed on a TSQ Quantum Discovery Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with an ESI source operating in positive mode. The ESI–MS operating variables used in this study were as follows: capillary voltage, 3.5 kV; source temperature, 350 °C; sheat gas pressure, 30 psi; auxiliary gas pressure, 10 psi; tube lens offset, 84V.

Mass spectrometry characterization of desmopressin and related peptides was performed on an Exactive benchtop Orbitrap-based mass spectrometer (Thermo Scientific, Bremen, Germany) operating in positive HCD scan at 50 eV. The sheath gas was set to 60 (arbitrary units), the aux gas set to 30 (arbitrary units) and the capillary temperature set to 350°C. The capillary voltage and spray voltage were set to 30 V and 3 kV, respectively. The instrument was operated in full scan mode from m/z 60–1200 at 100,000 resolving power. The data acquisition rate was 1 Hz.

3.6 Disulfide bridge reduction

In order to get additional information on the MS behavior of desmopressin, reduction of the disulfide bridge (Mpa_1-Cys_6) was performed by adding 200 µl of dithiothreitol in 0.2 mM ammonium acetate buffer at pH=5.5 (both from Sigma-Aldrich) to readily prepared solutions of the peptide and incubating the resulting solution for 30 minutes at 56 °C.

3.7 Validation

In accordance with Eurachem validation guidelines[27], 10 human plasma samples, were spiked at different levels (5, 10, 25, 50, 100 pg/mL) to determine the LOD.

The detection limit was defined as the lowest level at which a compound could be identified in all 10 plasma samples with the diagnostic ions present with a signal-to-noise (S/N) ratio greater than 3 and a retention time difference of less than 0.1 min to the reference.

Selectivity was tested by analysing plasma samples spiked with desmopressin and its analogues at a concentration of 100 pg/mL.

The analogues included the endogenous hormones AVP and oxytocin, and the synthetic derivatives [deamino-Cys_1, Val_4, D-Arg_8]-vasopressin, [Lys_8]-vasopressin, and terlipressin.

Specificity was tested during the validation procedure in order to check the absence of endogenous interferences. The 10 blank plasma samples used for determining analytical performances of the method were extracted and analyzed as described above.

3.8 Extraction efficiency and matrix effect

To evaluate the extraction efficiency, the 10 negative plasma samples, used for the validation, were spiked with desmopressin at 50 pg/mL and processed together with non-spiked plasma samples. The extracts of the non-spiked plasma samples were spiked after evaporation and before HPLC injection. After analysis the obtained peak areas for the product ion at *m/z* 328.0 of the two sets of samples were compared.

Matrix effect was evaluated by comparing the peak areas (*m/z* 328.0) of the plasma samples spiked after extraction with a reference solution of desmopressin at 50 pg/mL, corresponding to a 100% recovery (0% matrix effect).

3.9 Excretion studies

7 plasma samples from patients who received desmopressin were used in the study. Four patients received desmopressin via intranasal (≈ 10 µg/dose), two orally (200 µg) and one intravenously (20 µg). Blood samples were immediately centrifuged and plasma was separated. When not directly analyzed, plasma samples were stored at <-20°C awaiting analysis.

4 Results and discussion

4.1 Mass spectrometry

Direct infusion of desmopressin and analogues was performed in order to determine the best mass spectrometry conditions for the design of the MRM method. A full scan MS analysis was first performed in order to identify the most abundant precursor ions and subsequently the collision energy was optimized for the most diagnostic fragments.

Desmopressin showed excellent ionization, due to the presence of an arginine residue (Arg_8), containing the guanidinium group with a high proton affinity. The double charged ion $[M+2H]^{+2}$ was observed as base peak for desmopressin (Figure 3.2) but also for the analogues. The single charged ion $[M+H]^{+}$ only achieved a relative abundance of 20%. The collision-induced dissociation of desmopressin base peak (*m/z* 535.5) led to the formation of several fragments. Fragment ions at *m/z* 328.0, *m/z* 120.0 and *m/z* 214.0 were selected as diagnostic. Several other product ions, also present in the spectrum, were also recorded during mass spectrometry characterisation, but they were not specific or not sensitive enough using a criteria of S/N >3 to be considered for reliable detection. The other vasopressin-related peptides presented similar ESI spectra. Also, the ion $[M+2H]^{+2}$ appeared as base peak in the full scan MS spectra of each peptide. Additionally, the peptides presented several common fragments, but different precursor ions and retention times were observed, therefore method selectivity was guaranteed. Results from infusion experiments for MS/MS optimization of the investigated compounds are summarized in Table 3.1.

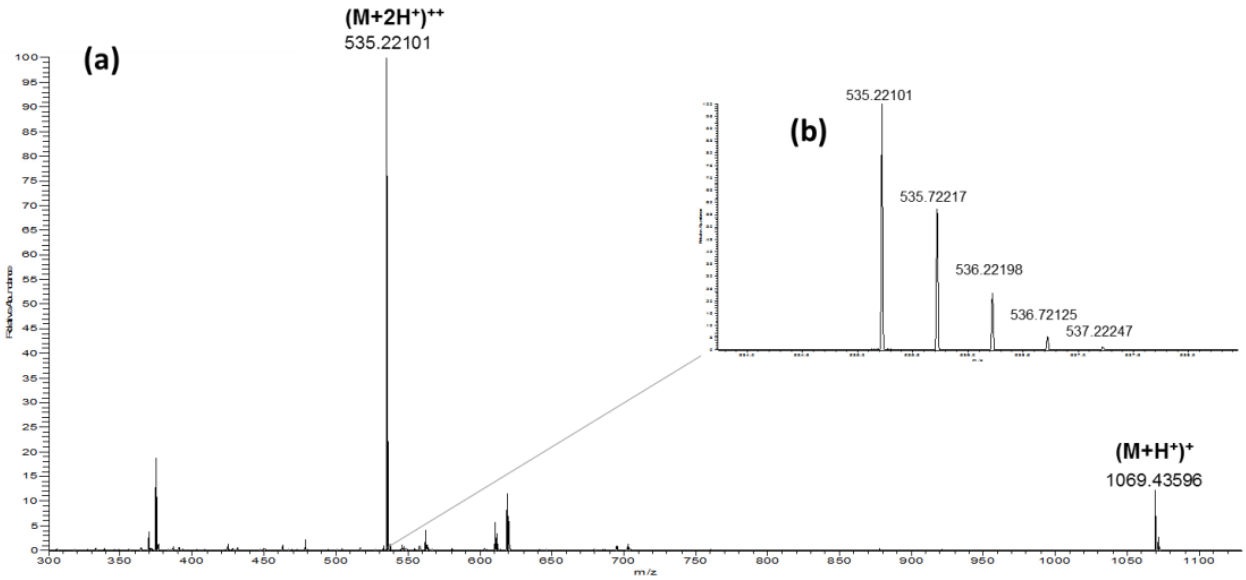


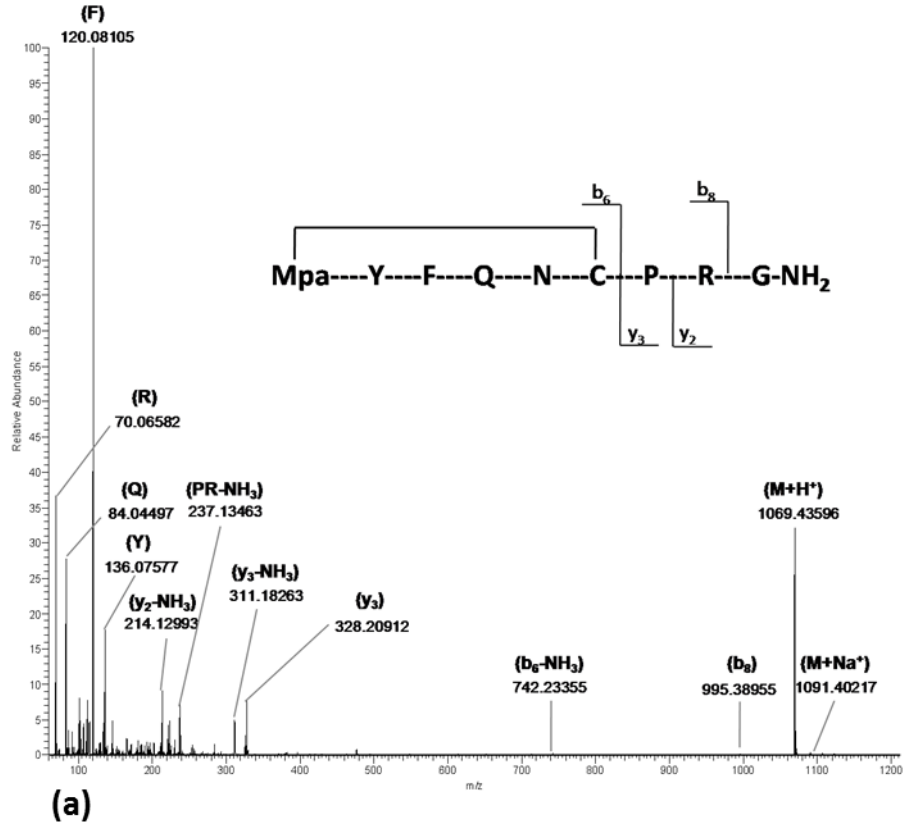
Figure 3.2 High-resolution full scan MS spectra of desmopressin (a), and isotopic pattern of its base peak $[M + 2H]^{2+}$ (b).

Table 3.1 Amino acid sequence, retention times and MRM conditions for ESI-MS/MS analysis of desmopressin and related peptides. Desmopressin and its analogues present several common product ions, but different retention time and parent ion. The double charged ion resulted to be the most abundant for all the investigated peptides.

Peptide	MW (Da)	Amino acid sequence	tR	Precursor ion charge state	Precursor ion m/z	Product ion (m/z)	Collision E (eV)	Abundance %
Arginine vasopressin	1083,4378	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2	3,6	$[M+2H]^{2+}$	542,7	120,0	37	100
						328,0	18	99
						757,3	14	24
Oxytocin	1006,4364	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	9,8	$[M+2H]^{2+}$	504,3	85,9	19	100
						294,9	37	62
						136,0	40	60
Desmopressin	1068,4269	Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-DArg-Gly-NH_2	9.9	$[M+2H]^{2+}$	535,5	328,0	16	100
						120,0	35	70
						214,0	16	13
Lys-vasopressin	1056,2200	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2	3,5	$[M+2H]^{2+}$	528,9	120,0	35	100
						128,9	34	35
						226,0	33	32
Terlipressin	1226,4961	Gly-Gly-Gly-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2	3,7	$[M+2H]^{2+}$	614,5	120,0	34	100
						128,9	36	99
						226,0	28	93
[deamino-Cys1, Val4, d-Arg8]-vasopressin (ISTD)	1039,4367	Mpa-Tyr-Phe-Val-Asn-Cys-Pro-DArg-Gly-NH_2	10,7	$[M+2H]^{2+}$	520,8	328,0	16	100
						120,0	34	67
						924,5	12	12

The three diagnostic ions selected for MRM method were further investigated by high-resolution mass spectrometry. Figure 3.3 shows the fragmentation patterns of the native (a) and the reduced (b) peptide generated by HCD at 50 eV at high resolution. All the relevant fragments are single-charged and some of them present a neutral loss of NH_3. Moreover, it is possible to notice that in the native molecule all the significant high-mass fragments (y_2-NH_3, y_3-NH_3, y_3, b_6) contain the Arg_8. The b_6-NH_3 fragment, which presents an intramolecular disulfide bridge between C1 and C6 and is adjacent to a proline residue, represents the only exception[28]. Reduction of the disulfide bridge provided additional information on the MS characterization of desmopressin, yielding to the identification of the complete y_3-y_7 sequence.

The contemporary presence in fragment ion y_3 of Arg_8 and Pro_6 at the N-terminal, which usually causes enhanced fragmentation attributed to the greater basicity of proline substituted nitrogen group[29], can explain the elevated abundance of this fragment in the MRM mode.



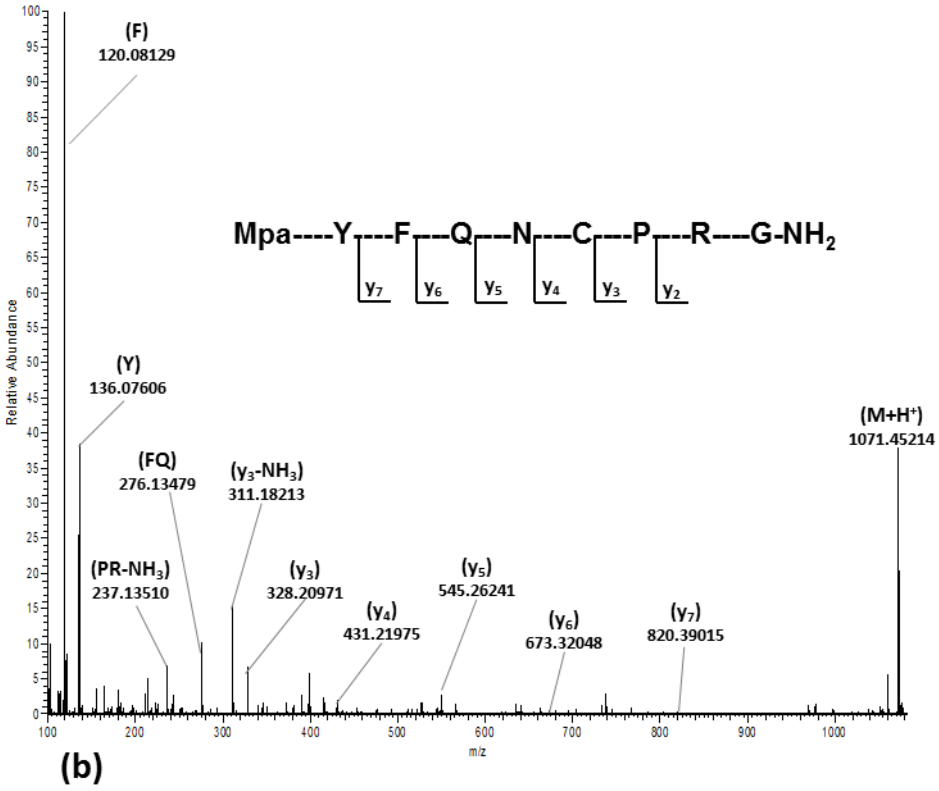


Figure 3.3 HCD spectra of desmopressin acquired at 50eV (a). The fragmentation scheme shows that all the most abundant high MW fragments contain the arginine residue, except

b_6, that is stabilized by the disulfide bridge. Reduction of the disulfide bridge (b) yield to the identification of the sequence y_3-y_7.

4.2 Liquid chromatography

Chromatographic separation was achieved with a C18 column, which allowed satisfactory peak shapes and chromatographic retention for desmopressin, the internal standard and the carrier peptide. A high percentage (90%) of ACN was required to elute desmopressin, which presented an average retention time (tR) of 10.07, as well as for the IS (tR=10.75 min). The carrier peptide AVP that contains the terminal amino moiety eluted earlier (tR=3.63 min). Additionally, other desmopressin related peptide, including the endogenous hormone oxytocin and the synthetic analogues [Lys_8]-vasopressin, and terlipressin were analyzed to further investigate method selectivity. Results, also summarized in Table 3.1, demonstrated that the chromatography is sufficient to separate desmopressin from these peptides. In fact, oxytocin elute at 9.88 min, [Lys_8]-vasopressin at 3.50 min, and terlipressin at 3.76 min.

4.3 Sample preparation

Since no LC-MS based method for detection of desmopressin in plasma has been described previously, the sample preparation procedures available from the literature were all related to the function of immunoassays. These approaches are based mainly on previous work of Lundin *et al*.[21,22]. Desmopressin is a small, highly basic peptide, resulting in good solubility, without a significant tertiary structure that can undergo denaturation during protein precipitation. Therefore Lundin *et al*.[21] successfully used protein precipitation with ice-cold acetone as sample clean-up. To optimize this protein precipitation step, the application of acetone, methanol, saturated $(NH_4)_2SO_4$ and ethanol[30] were evaluated during method development. Deproteination of the samples using saturated $(NH_4)_2SO_4$, gave the best results regarding S/N response. Additionally, omitting the use of an organic solvent to precipitate bulk proteins avoids the requirement to evaporate the deproteinated sample before application to the SPE in the next step. Finally, the use of protein precipitation allowed a 5-fold increase of the MS response compared with samples that were processed only with SPE. To further decrease sample preparation time, 15 and 30 min centrifugation instead of 60 min was considered. However, due to the lower centrifugation time,

deproteination was less complete. Using centrifugation devices with higher speed can further shorten centrifugation times.

SPE was considered to further concentrate and desalt the samples. In particular, cation exchange columns were investigated, because desmopressin is a basic peptide with a pKa value of approximately 12. Mixed-mode ion-exchange (MCX) columns were initially investigated. However, desmopressin could not be recovered from this type of column during the elution step since this type of column is only applicable for weak basic compounds.

Weak-cation ion exchange (WCX) columns proved to be particularly suitable for the clean-up of strong basic compounds allowing, compared to octadecyl (C18) and hydrophilic-lipophilic balance (HLB) sorbents, to eliminate efficiently acid and slightly basic matrix components. These resulted in a higher grade of purification.

Since the WCX protocol requires a basic pH to load the samples on the SPE column, the $(NH_4)_2SO_4$ saturated solution was basified with NH_4OH (pH 9.2). No differences in desmopressin responses were registered for protein-precipitation compared with the non-basified $(NH_4)_2SO_4$ saturated solution. The presence of 20% water in the elution solvent, resulting in a longer evaporation time, guaranteed the best results in terms of sensitivity.

4.4 Choice of internal standard and carrier peptide

[deamino-Cys_1, Val_4, D-Arg_8]-vasopressin was chosen as internal standard since it presents a very similar chemical structure, pKa, and retention times ($\Delta tR \approx$ 0.7 min) to desmopressin, and therefore a similar behavior during the entire analytical process. Since the degree of adsorption of a peptide or protein is not always predictable, several precautions were adopted to prevent this phenomenon, including the use of low binding plastic and the use of a carrier peptide. AVP was considered as a suitable carrier peptide for working solutions since it presents a very different retention time ($\Delta tR \approx$ 6.4 min) from the target analyte, which means no interferences with its detection, and, additionally, a low cost for the standard reference. We registered only a slight decrease (approximately 10´%) in MS response when diluted working solutions of 10 ng/mL of desmopressin, not containing AVP as carrier peptide, were analyzed, whereas no significant differences were recorded when

AVP was added to plasma sample (500 ng/mL) at the beginning of the sample preparation. Hence, desmopressin adsorption did not occur or was negligible during sample preparation.

Additionally, stability of desmopressin working solution stored at 4°C was tested by preparing a new solution (10 pg/mL) every month and comparing it with the previous by LC-MS analysis. After 4 months, no significant difference was noticed in peak areas of the various solutions. Moreover, desmopressin working solution was demonstrated to be stable after incubation at 56°C for 6 hours.

4.5 Method validation

The method showed an LOD of 50 pg/mL, with a signal to noise ratio (S/N) greater than 3 for the three transitions in all plasma samples that were analyzed. Maximum tolerance windows (% of base peak *m/z* 328.0) were set at ±10% (absolute) for *m/z* 120.0 and ±5% (absolute) for *m/z* 214.0, according to the WADA identification criteria for LC-MS qualitative assays[31] that recommend the use of minimum two transitions for MRM-based methods. Nevertheless, using only one transition is also allowed. According to the abundance recorded using the product ion at *m/z* 328.0, the average extraction efficiency (n=10) of the preparation was estimated at 40% (±4%). Signal reduction due to ion suppression in the ESI source (n=10) was limited to 5% (±14%).

The method also exhibited good selectivity since the related peptides presented different retention times, as shown in Table 3.1. Mass overlapping for mass spectrometry detection was avoided, thereby ensuring analytical selectivity. AVP, [Lys_8]-vasopressin, and terlipressin, whose N-terminal is not deaminated, elute earlier (3.5-3.80), whereas desmopressin, [deamino-Cys_1, Val_4, D-Arg_8]-vasopressin (both deaminated) and oxytocin (which presents the hydrophobic amino acid isoleucine) require a higher percentage of organic solvent.

The method can be used for the analysis of an elevated number of samples per batch and the whole analysis can be performed in a single day, since the only time consuming step (evaporation of the SPE eluate) requires only few hours.

4.6 Method application

The suitability of our method was evaluated by the analysis of 7 plasma samples from patients who received desmopressin. Four patients received desmopressin intranasally, two

orally and one intravenously. Administered dose and collection times are summarized in Table 3.2. Detection of desmopressin after oral administration could not be achieved, even taking into account only the most abundant transition (*m/z* 535 -> 328, LOD= 10 pg/mL). Indeed, orally administered desmopressin has a very low bioavailability. Osterberg *et al*.[19] reported maximum plasma levels < 50 pg/mL (t= 90 min) after oral administration of a therapeutic dose of 240 µg. Similarly, desmopressin was not detectable after nasal administration. The lower dose (≈ 10 µg) and the generally lower bioavailability of this type of formulation could explain the non-detection. After intravenous administration of a 20 µg dose, desmopressin could be detected in an unambiguous way (Figure 3.4b).

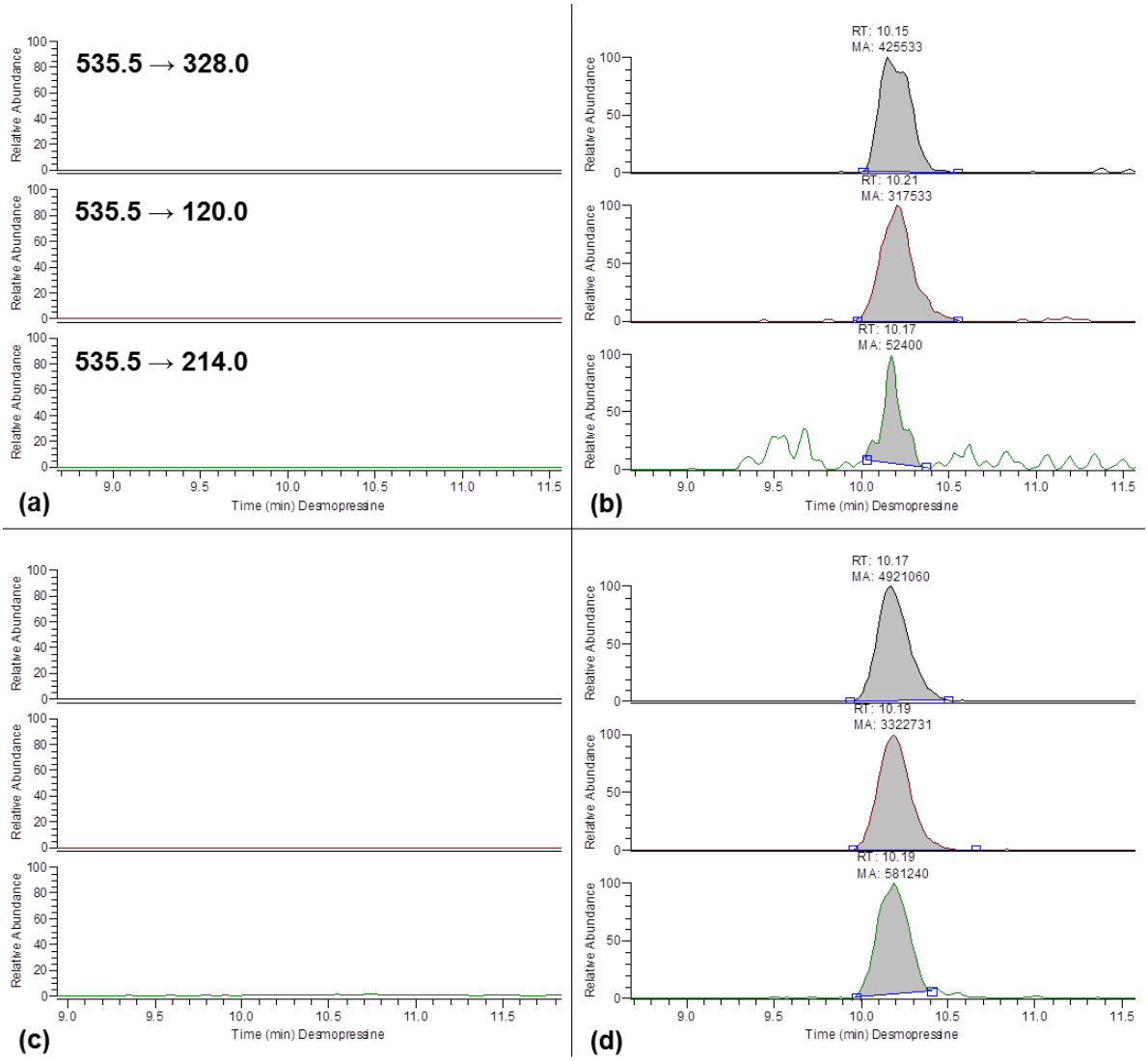


Figure 3.4 Extracted ion chromatograms for the product ions *m/z* 328.0, *m/z*_120.0, *m/z* 214.0 (precursor ion *m/z* 535.5) respectively in a blank plasma (a), after fortification with

desmopressin at LOD concentration (50 pg/mL) (b), before (c) and after administration of intravenous desmopressin (d). Samples were all prepared and analyzed according to the described assay.

Patient	Route of administration	Dose (µg)	Time of sample collection (h)	Detection
1	oral	200	2.00	No
2	intranasal	25	3.30	No
3	intranasal	25	3.00	No
4	intranasal	25	3.00	No
5	intravenous	20	0.30	Yes
6	oral	200	0.30	No

Table 3.2 Summary of blood specimens from excretion study, collected after administration of desmopressin via different routes.

It can be concluded that desmopressin detection in plasma related to anti-doping analysis is not realistic after oral or intranasal administration. In particular, since oral administration has shown to affect hematocrit levels[12] and intravenous application seems unlikely due to its invasive nature, it will be necessary to consider other detection strategies to increase the possibility to detect the misuse of this peptide by LC-MS. According to Agerso *et al.*[20], an important fraction of desmopressin is eliminated from the body via renal clearance. Hence urinary detection of desmopressin will be further investigated.

5 Conclusions

For the first time a sensitive LC-ESI-MS/MS method for the detection of desmopressin in human plasma for doping purposes was described. Validation of the method showed an LOD of 50 pg/mL when three transitions were considered, and 10 pg/mL when only the most abundant transition (*m/z* 328) was monitored. The method exhibited also good selectivity

and specificity. Application of the method to administration samples showed that desmopressin was detectable when administered intravenously, but not after oral and intranasal administration. The results of the analysis of the administration samples are in agreement with the literature describing low bioavailability and short plasmatic half-lives after oral and intranasal application, hampering its detection.

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Chapter 4

Detection of desmopressin in urine by liquid-chromatography-tandem mass spectrometry

Adapted from:

S. Esposito, K. Deventer, G. T'Sjoen, A. Vantilborgh, P. Van Eenoo. Doping control analysis of desmopressin in human urine by LC-ESI-MS/MS after urine delipidation. *Biomed. Chromat.* **2013,** 27(2), 240-5.

1 Abstract

The World Anti-Doping Agency (WADA) has recently added desmopressin, a synthetic analogue of the endogenous peptide hormone arginine vasopressin, to the Prohibited List, due to the potential masking effects of this drug on hematic parameters useful to detect blood doping.

A qualitative method for detection of desmopressin in human urine by high performance liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) has been developed and validated. Desmopressin purification from urine was achieved by means of delipidation with a 60:40 di-isopropyl ether/n-butanol and solid phase extraction with WCX cartridges.

The lower limit of detection was 25 pg/mL. Extraction recovery was determined as 59.3% (SD= 29.4), and signal reduction due to ion suppression was estimated to be 42.7% (SD= 12.9). The applicability of the method was proven by the analysis of real urine samples obtained after desmopressin administration via intravenous, oral and intranasal, achieving unambiguous detection of the peptide in all the cases.

2 Introduction

Desmopressin (1-desamino-8-D-arginine-vasopressin, dDAVP) is a synthetic derivative of the naturally occurring peptide hormone arginine vasopressin (AVP)[1,2], developed with the aim of having a longer lasting and more potent antidiuretic effect than AVP[2]. Recently, it has been demonstrated that the oral administration of desmopressin after EPO intake provokes rapid hemodilution, which causes the hematocrit to decrease[3,4]. In doping control analysis, hematocrit is an indirect marker of erythropoietins (EPOs) misuse and blood doping[5].

For this reason, the World Anti-Doping Agency (WADA) has added desmopressin to the Prohibited List as a masking agent and, consequently, methods for detection of desmopressin need to be developed to control its misuse[6]. Several detection methods using immunoassays for plasma and urine have been described[7–11]. These methods are very sensitive, having limits of detection (LODs) in the low pg/mL range, but they do not always provide the high grade of specificity and selectivity necessary in doping control analysis.

Our group developed a LC-MS/MS method for detection of desmopressin in plasma[12]. Although the method was sensitive (lower limit of detection: 50 pg/mL), it allowed only the detection of desmopressin after intravenous injection, but not for the oral and intranasal formulations, probably due to the low bioavailability of the drug when administered by these two routes[8]. According to Agerso *et al.*[9], an important part of the administered dose is eliminated by renal excretion. Recently, the detection of desmopressin in urine after intranasal and oral administration by using a hybrid quadrupole/time-of-flight (Q-TOF) mass spectrometer was described[13] showing good sensitivity. In the present study, a liquid chromatography-triple quadrupole tandem mass spectrometry method to detect desmopressin in urine is presented and was applied to the detection of desmopressin after intravenous, oral and intranasal administration. To improve the detection limit of the method, delipidation of the urine samples was also investigated.

3 Materials and methods

3.1 Chemicals and reagents

All the chemicals and solvents used for sample pretreatment and chromatography were analytical grade or HPLC grade. Desmopressin was a kind gift from Ferring Pharmaceuticals (Malmö, Sweden). AVP and [deamino-Cys_1, Val_4, D-Arg_8]-vasopressin were purchased from Sigma Aldrich (Saint Louis, USA). All the peptides were available as acetate salts. Stock and working solutions were all prepared using aqueous solutions containing 2% acetic acid (HOAc). Acetonitrile (ACN) and water (H_2O) were purchased from BioSolve (Lexington, USA); methanol (MeOH) was purchased from Fisher Scientific (Aalst, Belgium); 25% ammonia (NH_4OH) aqueous solution, ammonium chloride (NH_4Cl) and glacial HOAc were purchased from Merck (Darmstadt, Germany); trifluoroacetic acid (TFA), di-isopropyl ether and n-butanol were purchased from Sigma-Aldrich.

Ammonia buffer solution was prepared by dissolving 40 g of NH_4Cl in 100 mL of H_2O at room temperature. The resulting oversaturated solution was gently poured into a second glass bottle in order to get rid of the undissolved crystals of NH_4Cl, and then NH_4OH were added to the NH_4Cl solution until the final pH of 9.5 was reached.

Only low binding 1.5 mL vials (Eppendorf, Hamburg, Germany) and low retention pipette tips (Sorenson Biosciences, Salt Lake City, USA) were utilized to store stock solutions (100 µg/mL) and to prepare working solutions (10 ng/mL). AVP was always added to desmopressin working solutions at a concentration of 500 ng/mL to prevent desmopressin adsorption to solid surfaces.

3.2 Sample preparation

3 mL of urine were spiked with 2 ng of [deamino-Cys_1, Val_4, D-Arg_8]-vasopressin as internal standard. The samples were then acidified (pH= 3.5-4.0) by adding 500 µl of 5% HOAc aqueous solution. Samples were then delipidated by adding 5 mL of di-isopropyl ether:n-butanol 60:40 solution, vortexing (30 seconds) and then centrifuged (4100 g, 20 minutes, 5°C). The upper layer was discarded and a second delipidation step with di-isopropyl ether:n-butanol 60:40 was performed at the same conditions but with a basic pH (9.5-10.0) after

addition of 500 µl of ammonia buffer. After centrifugation, the upper layer was discarded again and the remaining organic solvent was rapidly eliminated by evaporation under a gentle nitrogen stream (5 minutes, 30°C). The delipidated urines were then further purified by solid phase extraction (SPE) with Oasis® WCX (60 mg) cartridges, purchased from Waters (Milford, USA) with a procedure derived from our previous work on plasma. The eluate was subsequently evaporated to dryness with a centrifugal evaporator (45°C, 240 g, 6-8 h), and then dissolved in 40 µl of 95:5:0.1:0.01 H_2O:ACN:HOAc:TFA prior to LC-MS analysis.

3.3 LC-MS

The samples were analyzed using the same LC-MS conditions as described in the previous work in plasma (Chapter 3). HPLC separation was achieved with a Surveyor MS Pump Plus coupled with a Surveyor Plus autosampler (Thermo Scientific, Bremen, Germany) using a Zorbax 300SB-C18 reverse-phase column (1.0 x 50 mm, 3.5 µm) protected with a Zorbax 300SB-C8 guard column, both from Agilent Technologies (Santa Clara, USA).

A binary gradient was used: mobile phase A consisted of H_2O, 0.1% HOAc, 0.01% TFA; mobile phase B consisted of ACN, 0.1% HOAc, 0.01% TFA. Gradient elution was as follows: 95% A for 1.5 minutes, then decreased linearly to 0% A in 8.5 minutes, and held at 0% A for 5 minutes, followed by an increase to 95% A in 0.1 minutes. Then the system was equilibrated for 10 minutes before next injection (total run time: 25 minutes). A constant flow rate of 50 µl/min was maintained. For each sample, 30 µl were injected.

MS/MS detection was performed on a TSQ Quantum Discovery Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with an ESI source operating in positive mode. The ESI–MS operating variables used in this study were as follows: capillary voltage, 3.5 kV; source temperature, 350 °C; sheat gas pressure, 30 psi; auxiliary gas pressure, 10 psi; tube lens offset, 84V.

3.4 Validation

In accordance with Eurachem validation guidelines[14], 10 different blank human urine samples, were spiked at different desmopressin levels (10, 25, 50, 100, 200 pg/mL) to determine the LOD. The detection limit was defined as the lowest level at which a compound could be identified in all 10 urine samples with both the diagnostic ions present with a

signal-to-noise (S/N) ratio greater than 3 and a retention time difference of less than 0.1 min with the reference, as recommended by WADA criteria for qualitative assays[15].

Specificity was tested during the validation procedure. The 10 blank urine samples used for determining detection capability were extracted and analyzed as described above.

3.4 Extraction recovery and matrix effect

To evaluate the extraction recovery, the 10 negative urine samples, used for the validation, were spiked with desmopressin at 50 pg/mL and processed together with non-spiked urine samples. The extracts of the non-spiked urine samples were spiked after evaporation and before HPLC injection. After analysis the obtained peak areas of the two sets of samples were compared.

Matrix effect was evaluated by comparing the peak areas of the urine samples spiked after extraction with a reference solution of desmopressin at 50 pg/mL, corresponding to a 100% recovery (0% matrix effect).

3.5 Stability

Urine samples fortified with desmopressin at 1 ng/mL were stored at room temperature and at -20°C. 3 mL specimens were collected after 0, 1, 2, 3, 4 and 5 days and stored at -20°C until analysis.

3.6 Excretion studies

Six urine samples from patients who received desmopressin were used in the study. Samples were obtained from the Department of Internal Medicine (Endocrinology) and Hematology of the Ghent University Hospital, with the approval of the Ethical Committee of the Ghent University (reference: B67020108809). Three patients received desmopressin intranasally (≈ 10 µg/dose), two orally (200 µg) and one intravenously (20 µg). Samples were stored at -20°C awaiting analysis.

4 Results and discussion

4.1 Liquid chromatography

Chromatographic separation was achieved by using a C18 column, which allowed satisfactory peak shapes and chromatographic retention for desmopressin, the internal standard and the carrier peptide. Using this column, a high percentage (90%) of ACN was required to elute desmopressin and the IS. The carrier peptide AVP that contains the terminal amino moiety eluted earlier (Figure 4.1).

Different concentrations of HOAc and TFA acid in the aqueous and the organic mobile phases (from 0.01% to 1%) were tested during method development. The intensity of the chromatographic peak for desmopressin was higher in case of using 0.1% HOAc, 0.01% TFA for both the mobile phases.

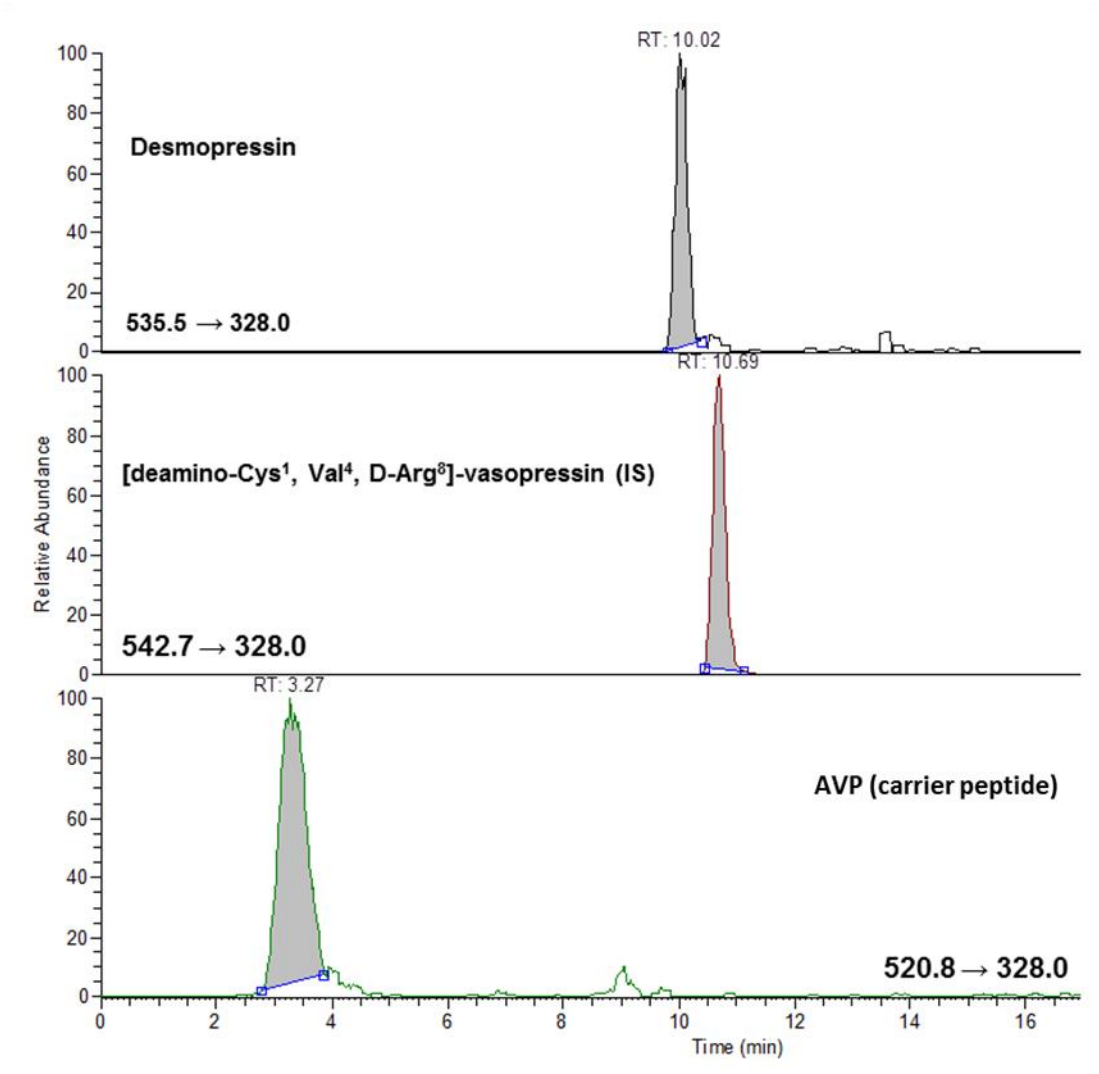

Figure 4.1 Multiple reaction monitoring (MRM) extracted ion chromatograms for desmopressin of product ion at *m/z* 328.0 for desmopressin (parent ion at *m/z* 535.5), AVP (parent ion at *m/z* 542.7) and the internal standard (parent ion at *m/z* 520.8).

4.2 MS/MS optimization

The full scan mass spectrum of desmopressin exhibited an abundant $[M + 2H]^{2+}$ ion (Figure 4.2a), whereas the monocharged pseudomolecular ion $[M + H]^{+}$ has approximately half of the intensity. Hence the double charged species was used as precursor to generate product ions. The product ion spectrum for this peptide showed several ions (Figure 4.2b) with good intensities (*m/z* 328.0, 120.0, 214.0, 276.1, 430.4 and 526.7). The origin of these fragments has been explained previously[12].

Two precursor-product ion transitions were selected for the MRM (*m/z* 328.0, 120.0 from parent ion *m/z* 535.5), according to the identification criteria for mass spectrometry-based qualitative assay recommended by WADA. For the IS and the carrier peptide only the most abundant transition was selected (respectively *m/z* 328.0 from parent ion *m/z* 542.7, and *m/z* 328.0 from parent ion *m/z* 520.8). However, the carrier peptide was not detected in the optimized method, due to the use of the divert valve.

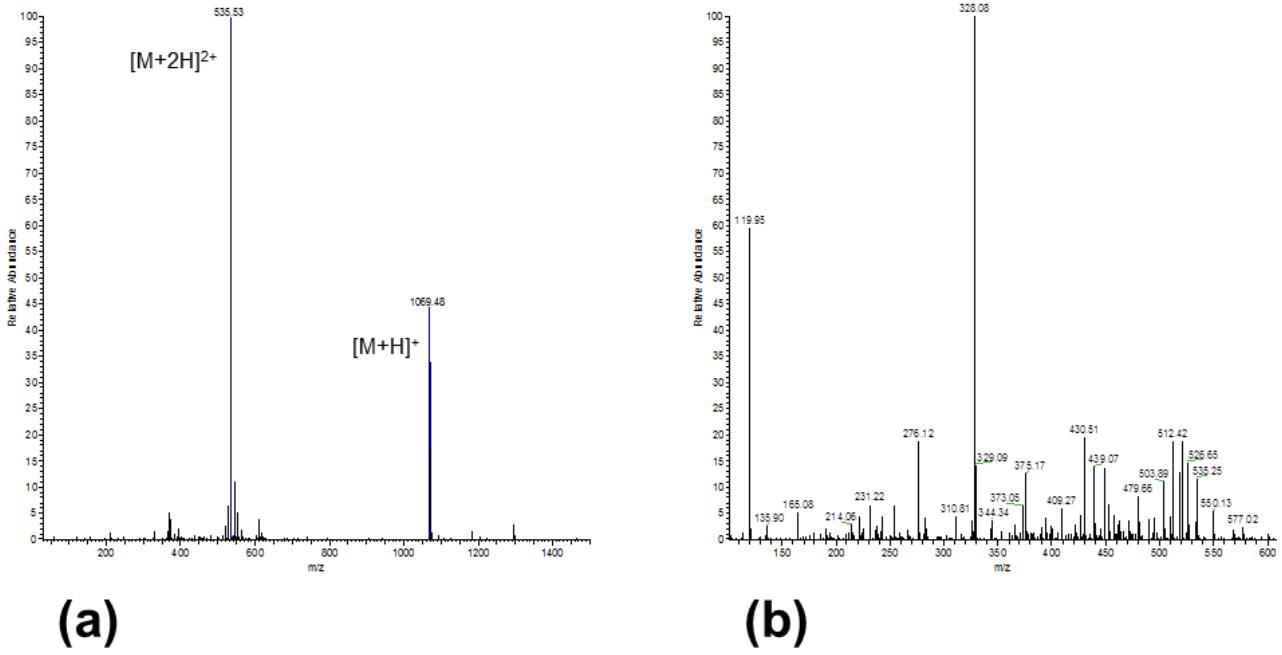

Figure 4.2 Desmopressin full scan MS (a) and product ion scan (b) of the parent ion $[M + 2H]^{2+}$.

4.3 Sample preparation

The majority of sample preparation protocols described in literature is optimized for immunoassays. Agersø *at al*.[9] successfully used SPE with octadecylsilyl cartridges as sample clean-up prior to radioimmunoassay. The procedure was based on previous work of Lundin *et al*.[7] who, in turn, adapted a method from Tausch *et al*.[16] for the detection of urinary AVP.

Cation exchange columns have proven to be useful for the specific detection of desmopressin[12,13] and have proven to result in cleaner extracts than the octadecylsilyl cartridges. Indeed, using the WCX cartridges protocol as a stand-alone sample preparation technique; the product ion at *m/z* 328.0 was detectable at 100 pg/mL. Although, according to the WADA criteria for identification, one precursor-product ion pair is sufficient for identification, the use of two precursor-product ions is recommended. Unfortunately, the second most abundant ion at *m/z* 120.0 was highly interfered (Figure 4.3a). To obtain this latter transition with improved sensitivity and specificity, a further purification step was needed in addition to the WCX cartridge. Since these interferences were eluted from the SPE-column together with the desmopressin and coeluted during the chromatographic method also with desmopressin, they were considered to be from apolar origin. Hence, the use of an orthogonal technique was considered to eliminate these non-polar interferences and improve sample clean-up. A previous method for plasma delipidation without protein denaturation was adapted[17]. The adapted procedure consists of a liquid-liquid extraction of the lipids with a mixture of di-isopropyl ether:n-butanol 60:40 at a volume ratio of 2:1, pH 5.2 and the other extraction was performed at pH 9.5 to remove both acidic and basic interferences. Due to the high pKa of the guanidine group in the arginine, desmopressin is in ionized form in the pH range used for the delipidation. Hence, desmopressin remains in the aqueous phase.

Despite the fact that lipids are less abundant in urine than in plasma, a relevant effect was noticed, as presented in Figure 4.3b. The sample delipidated before SPE clearly showed a better signal for both transition compared to the same sample purified only with SPE. The increase in response for the product ion at *m/z* 328.0 was moderate but still relevant (approximately 30% as peak area and +50% as signal-to-noise ratio), whereas the quality of

the peak chromatogram was dramatically improved for the product ion at *m/z* 120.0, allowing to have an additional diagnostic ion, as recommended by WADA.

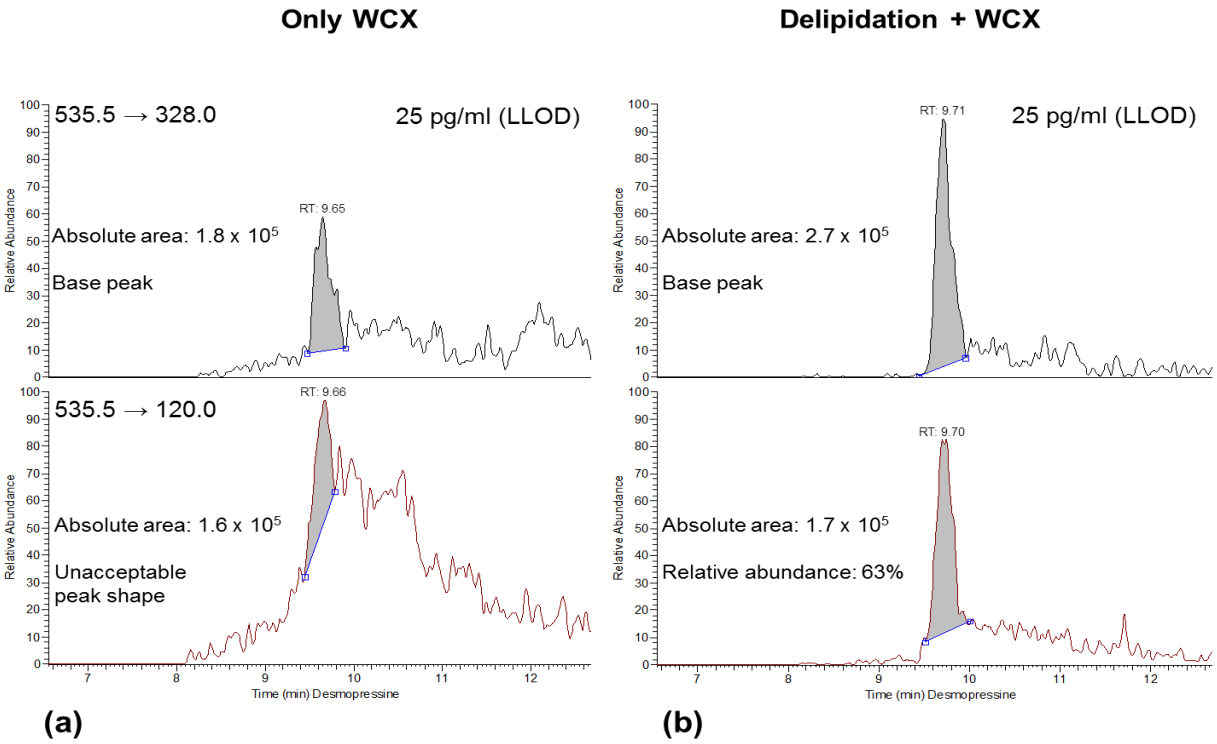

Figure 4.3 MRM extracted ion chromatograms of the product ions at *m/z* 328.0 and *m/z* 120.0 from the parent *m/z* 535.5 for a urine sample fortified with desmopressin at the limit of detection processed only with WCX cartridges and with the combination of delipidation and WCX. The effect on MS response and peak quality is evident, particularly for the less abundant fragment.

4.4 Validation

Validation was conducted using the optimized conditions.. Finally, the method showed an LOD of 25 pg/mL, with signal to noise ratio greater than 3 for the two diagnostic transitions in all urine samples that were analyzed. Maximum tolerance windows (% of base peak *m/z* 328.0) were put ±10% (absolute) for *m/z* 120.0, according to the WADA identification criteria.

The signals for the extracted diagnostic ions were not interfered at the retention time of desmopressin from the ten urine sample analyzed during method validation. The internal standard and the carrier peptide presented different retention time and did not generate any interference.

[deamino-Cys_1, Val_4, D-Arg_8]-vasopressin showed to be an effective internal standard to control all the pre-analytical and analytical steps, with a sufficiently constant mass spectrometry response (RSD= 35.7%) and an absolute peak area $>10^6$ in all the samples analyzed.

4.5 Extraction recovery and ion suppression

MRM extracted ion chromatogram from a pure reference standard, a sample spiked prior to injection and a sample spiked before extraction were compared. According to the abundances recorded, the average extraction recovery of the preparation was estimated at 59.3% (SD= 29.4). Signal reduction due to ion suppression in the ESI source was calculated as 42.7% (SD= 12.9).

4.6 Demopressin stability

Desmopressin proved to be stable under freezing conditions in urine samples. At room temperature, however, some signs of degradation were noticed. After 5 days at room temperature, only approximately 80% of the initial dose was recovered. However, for the anti-doping framework storage at room temperature for such a period is not in agreement with the requirements set out in the International Standard for Laboratories[18]. Therefore this simulation experiment represents an extreme condition (worst-case scenario) that is not commonly present during routine sample handling.

Additionally, the working solutions, stored at 4°C, showed optimal stability when compared after 1, 2 and 3 months with freshly made solutions.

4.7 Method application

As shown by the extracted ion chromatograms of product ions at *m/z* 328.0 and 120.0 in Figure 4.4, desmopressin was unambiguously detected in all urine samples collected after administration of the formulations summarized in Table 4.1. These findings confirm the work of Agersø *et al*.[9], which described urinary concentration of desmopressin higher than in plasma. No explanation for this high urinary excretion rate has been described so far, but the ionized guanidine/arginine moiety in the structure of the peptide might have an important role in increasing renal clearance.

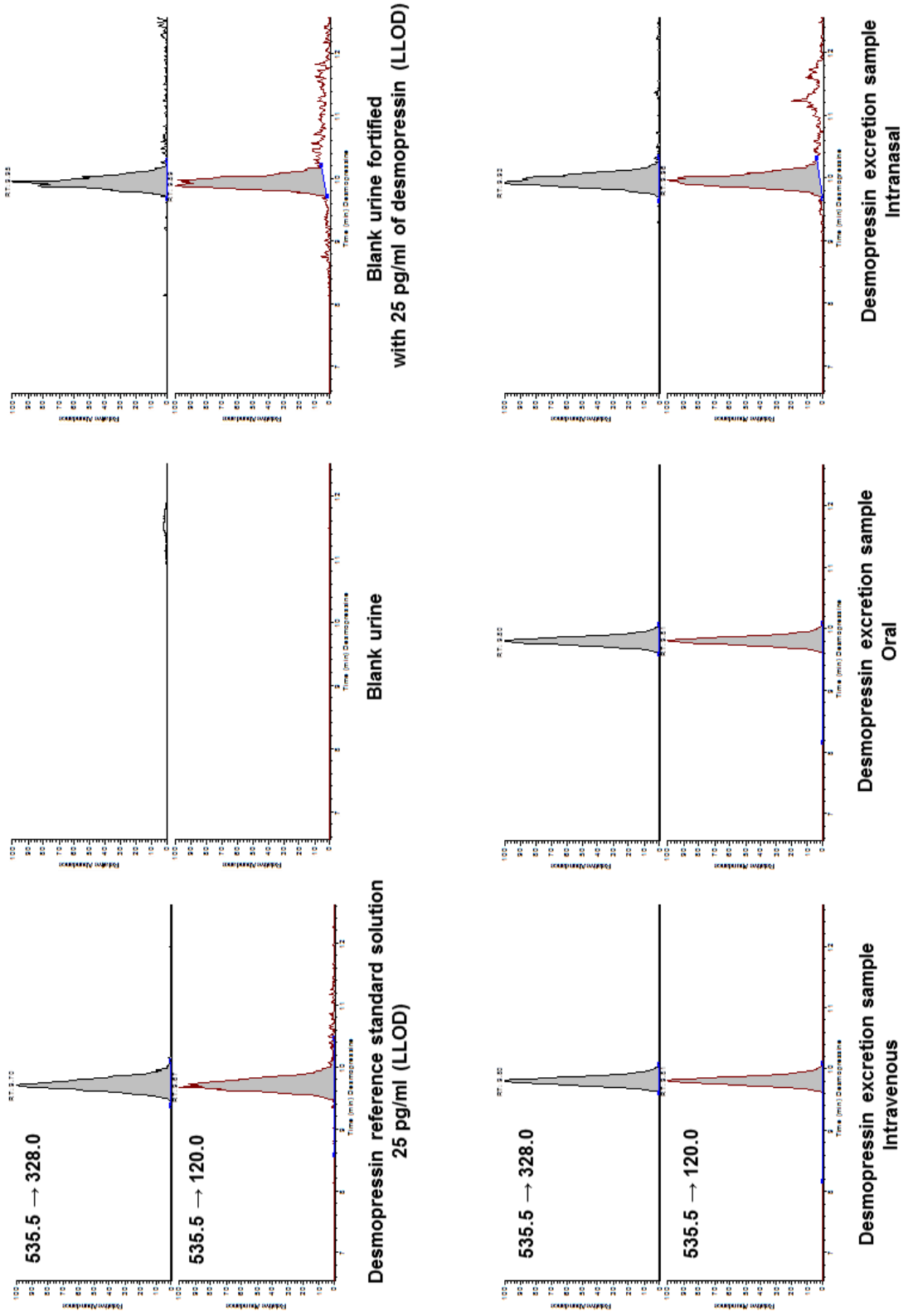

Figure 4.4 MRM extracted ion chromatograms of the diagnostic product ions at m/z 328.0 and m/z 120.0; desmopressin was unambiguously detected in urine after intravenous, oral and intranasal administration. No signal was registered in blank urine samples.

The high elimination rate from blood circulation of desmopressin and the low bioavailability of oral and intranasal formulations[9] were probably the main causes of the non-detection of this compound in plasma after oral and intra-nasal administration, as observed in our previous work[12]. In fact, as shown in the previous chapter, desmopressin was only detectable in plasma after intravenous administration. Urine sample collected from the same patients at the same post-administration time have revealed that urine must be considered the preferred detection matrix for doping control purposes, confirming the recent work of Thomas *et al*.[13].

Table 4.1 Summary of urine samples from excretion study, collected after administration of desmopressin via different routes

Patient	Route of administration	Dose (µg)	Time of sample collection (h)	Detection
1	oral	200	2.00	Yes
2	intranasal	25	3.30	Yes
3	intranasal	25	3.00	Yes
4	intranasal	25	3.00	Yes
5	intravenous	20	0.30	Yes
6	oral	200	0.30	Yes

5 Conclusions

A reliable LC-MS/MS method for detection of desmopressin in human urine has been successfully developed and validated. The method showed good sensitivity (LOD= 25 pg/mL), with the required specificity and selectivity.

In particular, the use of the double (acidic/basic) delipidation step of the urine samples improved the performance of the method making it a promising tool to improve urine clean-up for the urinary detection of other peptides as well.

The results of the excretion studies demonstrate that urine is the preferred matrix for the detection of desmopressin abuse after oral, intra-nasal or intravenous administration.

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Part III - Black market of prohibited peptides

Chapter 5

Peptides and black markets: a matter of doping and public health

1 Abstract

Peptide hormones are becoming increasingly appealing to amateur and professional athletes as performance enhancing drugs. A particular aspect of this trend is represented by black markets, where a clandestine, uncontrolled trade of these substances takes place. Control of this phenomenon involves surveillance, public health and sport testing authorities.

Several classes of peptides have reached the focus of being used as performance enhancing drugs: growth hormone-releasing peptides (GHRPs), growth hormone-releasing hormone (GHRH) analogues, insulin-line growth factor 1 (IGF-1) analogues, mechano-growth factors (MGFs), growth hormone fragments (AOD9604), luteinizing hormone-releasing hormone (LHRH) agonists, and β-thymosins.

Doping control analysis of these emerging drugs is a challenging task for laboratories. The numerous issues related to the misuse of black market peptides are analyzed here, including tools and strategies for identification of new peptide hormones, development of analytical methods and alternative models for pharmacokinetics (metabolism) studies, and public health issues. Finally, future perspectives for doping control laboratories are discussed.

2 Introduction

Different classes of peptides with different pharmacological properties have become popular among elite and amateur athletes and bodybuilders for their performance-enhancing or masking properties or to improve physical appearance. Several of these prohibited substances, such as insulins and desmopressin, have received approval from regulatory agencies and are included in Section S2 of the WADA Prohibited List ("Peptide hormones, growth factors and related substances"). Those that have not been approved are prohibited regardless of their doping effect (Section S0)[1]. These formulations are not available in legal markets; nevertheless they are finding their way onto parallel, illegal channels of distribution. Such markets have long been dominated by steroids[2,3], but popularity of peptides is increased as demonstrated by the amount of seizures documented in the last years[4–7]. This phenomenon implies difficult challenges and new approaches for doping control laboratories.

3 Black market peptides

Doping control authorities and laboratories are currently facing an ever-evolving list of peptides coming from the black market. To the best of our knowledge, seven main classes have been described so far, according to pharmacological properties and structures (Table 5.1). For some of them, the doping effect is only theoretical and has not been demonstrated yet. The majority of them, including growth hormone releasing peptides (GHRPs), growth hormone releasing hormone analogues, insulin-line growth factor 1 (IGF-1) analogues, mechano-Growth factors, and AOD9604, acts on the GH/IGF-1 axis.

Table 5.1 List of peptide classes which are reportedly available in black markets.

Class	Peptides	Approved for therapeutic use	References
GHRPs	(GHRP)-, -2, -4, -5, 6, hexarelin, alexamorelin, ipamorelin	Only GHRP-2	8-12
GHRHs	GHRH(1-44), CJC-1293, CJC-1295, sermorelin, tesamorelin, [Pro1,Val14]-GHRH(1-44)	Only tesamorelin	15,16
IGF-1 and analogues	IGF-1, IGF-1 LR3, IGF BP3	Only recombinant human IGF-1	2,17,18
MGFs	MGF 1Ec, N-term amidated MGF, PEG MGF	No	23
GH fragments	AOD-9604	No	26
GnRH and analogues	GnRH, leuprolide, buserelin, histrelin, goserelin, deslorelin, nafarelin, triptorelin	Yes	-
β-thymosins	Thymosin β4 (43 AA, N-term acetylated), TB-500 (AA 17-23, N-term acetylated)	No	31
Unknown	cyclo[Arg-Lys-N(C_6H_9)Gln-Phe]	No	32

GHRPs are the most investigated class of "small" prohibited peptides (molecular weight < 1.5 kDa)[8–12]. GHRPs are synthetic analogues of met-enkephalin, a peptide with opioid effects, which stimulate GH synthesis and have no effect on the opioid receptor. Only GHRP-2 has received therapeutic approval, but it is available also as black market formulations[10,11]. It has been demonstrated that GHRP-2 can produce indirect GH doping which cannot be detected with the GH isoform differential immunoassay[13].

GHRH, also known as growth-hormone-releasing factor (GRF), is a 44 amino acid (AA) hypothalamic peptide that stimulates GH production and release. Analogues have been developed both on the basis of the intact peptide (tesamorelin)[14] and on the 1-29 fragment (sermorelin, CJC-1295 and CJC 1295)[15,16]. Moreover, a novel analogue of GHRH(1-44) with an unusual sequence has been identified in a confiscated vial in our laboratory (Chapter 7).

IGF-1 (70 AA) mediates most of the anabolic effects of GH on protein metabolism, inhibiting protein breakdown and promoting protein synthesis. There are currently two approved preparations: Increlex (recombinant human IGF-1) and Iplex (IGF-1 bound to its binding protein IGFBP-3, but formulations containing IGF-1 and derivatives are also marketed illegally[2,17].

MGF is a 24 AA splice variant of IGF-1 which is expressed in mechanically overloaded skeletal muscle[18–20]. Its role in muscle regeneration after exercise-induced damage has been demonstrated *in vivo*[21]. A C-term amidated MGF identified in confiscated material is described in this study (Chapter 6)[22]. Additionally, formulations containing a not better described PEGylated MGF appear to be available on Internet, but there is no description of this analogue in the literature, and so far no case of identification have been reported.

The hexadecapeptide AOD9604 consists of the GH Fragment 177-191, modified with the addition of a tyrosine residue at the N-terminus. This portion of GH presents lipolytic action, and therefore AOD9604 has been investigated as anti-obesity drug[23]. Recently, it has been shown how this peptide does not affect the GH isoform immunoassay[24]. Furthermore, detection and *in vitro* metabolism have been recently investigated[25].

Pulsatile administration of luteinizing hormone-releasing hormone (LHRH) agonists may increase the secretion rate of luteinizing hormone and, consequently, testosterone[26,27]. Since LHRH has very short half-life, several analogues have been designed with AA substitutions at position 6 and/or 10[28]. Among these, Lupron (leuprolide), Synarel (nafarelin), Buserelin and Zoladex (goserelin) have received approval for treatment of prostatic cancer and pain associated with endometriosis[29]. LHRH and leuprolide have been identified in more than one case in a confiscated product by the Ghent Doping Control Laboratory (Table 5.2).

TB-500 has been identified as the N-term acetylated 17-23 fragment of thymosin β4 (Chapter 7), which corresponds to the actin-binding portion of the molecule and therefore it may as well have a role in tissue regeneration[30]. This compound is suspected to be used both in humans and in racehorses[31]. The intact form of thymosin β4 (44 AA) has also been reported.

Finally, a cyclic peptide with potential doping effect, corresponding to the sequence cyclo[Arg-Lys-N(C_6H_9)Gln-Phe], has been identified, but there is no description in literature of the function or the effects of this molecule[32].

The peptides which were identified in DoCoLab in confiscated products during the last three years are listed in Table 5.2. The list contains also non-doping related peptides such as melanotan (a tanning agent) and thymo pentin (an immunostimulant).

Table 5.2 List of peptides identified by DoColab in confiscated products in the last three years.

Peptide	Sequence
GHRP-2	(D-Ala)-(D-β-Nal)-Ala-Trp-(D-Phe)-Lys-N
GHRP-6	His-(D-Trp)-Ala-Trp-(D-Phe)-Lys-NH2
Ipamorelin	Aib-His-(D-2-Nal)-(D-Phe)-Lys-NH2
Hexarelin	His-(D-Mrp)-Ala-Trp-(D-Phe)-Lys-NH2
LHRH	Pyr-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2
Leuprolide	Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt
Triptorelin	pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2
TB-500	(Acetyl)Leu-Lys-Lys-Thr-Glu-Thr-Gln
full lenght Thymosin β4	(Acetyl)MSDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES
Amidated MGF	YQPPSTNKNTKSQRRKGSTFEERK-NH2
hGH (22 kDa)	FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSES IPTPSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLL KDLEEGIQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKD MDKVETFLRIVQCRSVEGSCGF
sermorelin/Geref	YADAIFTNSYRKVLGQLSARKLLQDIMSRQ
CJC-1293	Y(d-A)DAIFTNSYRKVLGQLSARKLLQDIMSR-NH2
CJC-1295	Y(d-A)DAIFTQSYRKVLAQLS ARKLLQDILSR-NH2
[Pro1, Val14]-hGHRH	PYADAIFTNSYRKVVLGQLSARKLLQDIMSRQQGESNQERGARARL
Melanotan II	*Ac-Nle-cyclo[Asp-His-D-Phe-Arg-Trp-Lys]-NH2*
Thymopentin	*Arg-Lys-Asp-Val-Tyr*

4 From discovery to detection

4.1 Identification of new peptides

A fundamental role in the discovery of new unapproved peptidic drugs is played by the collaboration between laboratories and surveillance authorities, such as Custom Agency, as demonstrated by several case reports involving confiscated material, which have also received media attention[4,6,7]. Another important source of information is represented by internet markets, where peptides can be easily purchased with few clicks, and "underground literature" coming from websites such as bodybuilding forums.

Identification of the active content of a black market formulation is the first step towards effective doping control analysis of a new doping agent. Several tools originally developed for large-scale proteomics studies have been used for the structural characterization of this type of molecules[33]. Particularly, liquid-chromatography coupled to high-resolution mass spectrometry (LC-HRMS) is the most used technique for this purpose, due to the possibility to obtain molecular mass of the compound via spectral deconvolution and to define the peptide sequence based on characteristic fragmentation pathways[34,35].

For small peptides, top-down approach is generally used[10,11,36]. With increasing the size of the peptide, bottom-up analysis including trypsin digestion can improve quality of the characterization[37], producing smaller peptides whose sequences can be combined to identify the molecule, as shown on Figure 5.1 for MGF. Although bigger proteins are not part of this study, it is worth mentioning that trypsin digestion is mandatory for identification of bigger proteins that ionize poorly when intact[38], and in case of glycosylated protein (e.g.: erythropoietins), enzymatic deglycosylation is also required[39]. For big proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is also an useful tool to establish molecular weight[38].

In a typical proteomics workflow, once the sequence has been elucidated, the identity of the protein/peptide can be confirmed by matching the observed sequence against sequence databases[40]. This can be applied also in the case of peptidic doping agents, especially if the identified peptide belongs to a newly discovered class of doping agents (e.g.: MGF[22]).

However, the creation of an internal database (containing sequences of native of digested analogues and constantly updated whenever a new species is identified) can significantly speed up the identification process in case of the discovery of new analogues of an already known class.

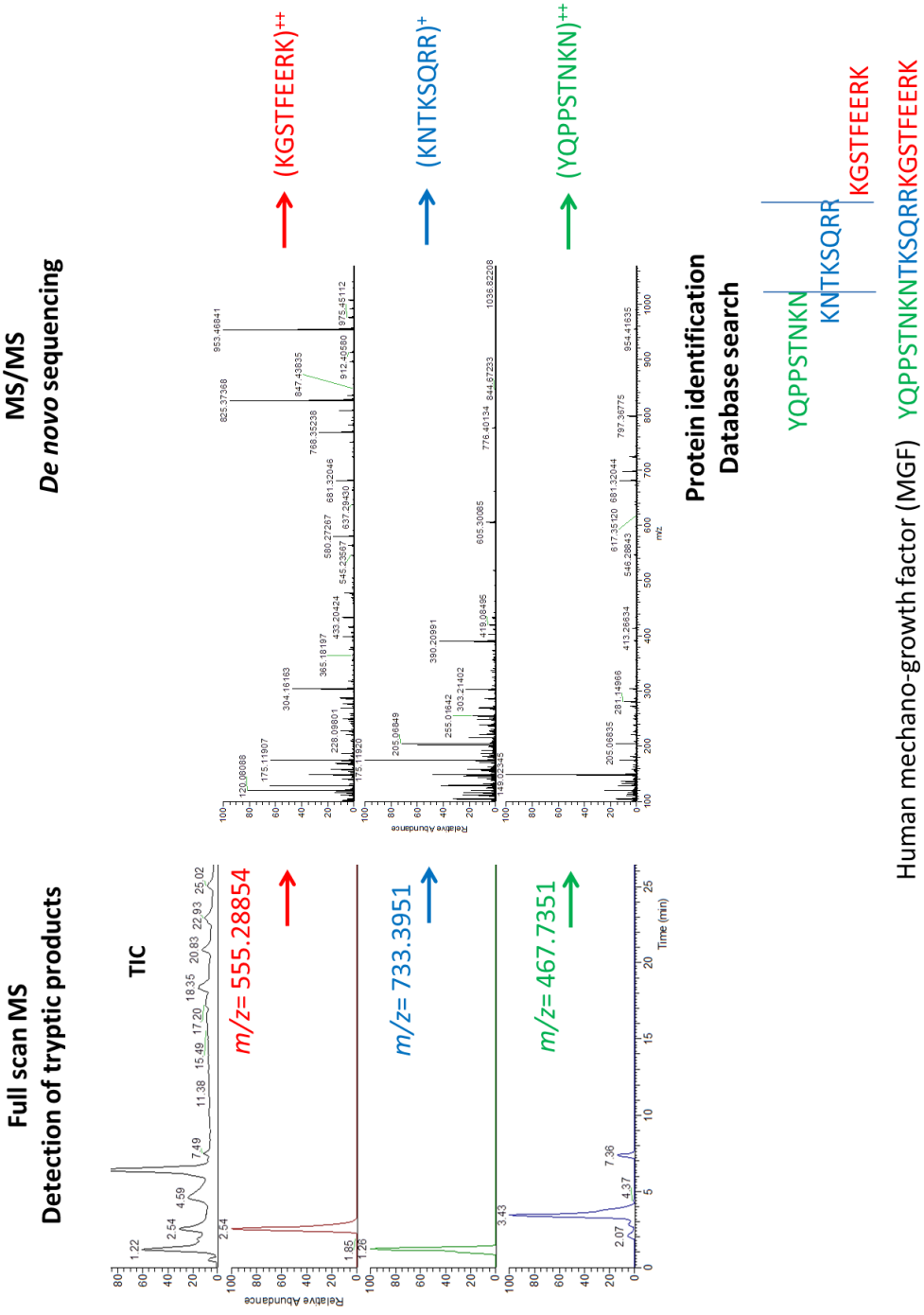

Figure 5.1 Example of *bottom-up* identification (human MGF).

Since some peptides, such as GHRPs, have D-amino acid, nuclear magnetic resonance (NMR) analysis is recommended to elucidate completely the stereochemistry of the molecule[11,36].

The first reports on black market peptides are dated 2010 and describe the identification of GHRP-2[10], hexarelin[2], His-tagged IGF-1 LR3[2,17], and CJC-1295[37]. More recently, new cases were recorded, including the unknown cyclic peptide confiscated during a horserace event[32], C-term amidated mechano-growth factor[41] identified in an unknown vial, and TB-500[36].

These papers already show the issues related to these cases: unlabelled or mislabelled vials[2,17,32,41], and presence of related impurities and/or unknown excipients[2]. From the point of view of doping control laboratories, the presence of non-characterized derivatives or impurities implies additional challenges for the development of effective LC-MS based methods.

4.2 Analytical methods

Since peptide hormones have generally very low plasma and urinary concentration (often in the femtomole range) and a short half-life (generally < < 1-2 days)[8,42], efficient testing of these substances is a difficult task, which becomes even more complicated in case of non-approved drugs. In fact, pharmacokinetics (PK) studies, useful to characterize metabolism and excretion, are in this case limited by ethical constraints. In absence of PK data, it is also difficult to evaluate the fitness for purpose of an analytical method, especially in terms of limit of detection and detection window. Additionally, there is often lack of reference standard material in the market. On the other hand, custom peptide synthesis is currently available on the market at competitive prices; therefore laboratories have the possibility to obtain high-purity standards to use for their methods.

Several LC-MS methods have been reported for the detection of peptides, which can be roughly divided according to the sample preparation strategies: solid-phase extraction (SPE)[12] and immunoaffinity purification (IAP)[43]. Urine is generally preferred to plasma for small peptides, because they tend to have longer detectability in urine (e.g.: desmopressin[44,45]). Nevertheless, as suggested by Thomas *et al.*[46], it could be particularly interesting to analyze plasma in the context of GH abuse, since the same serum samples collected for GH analysis could also be used to screen GHRPs.

IAP represents the technique of election for purification of bigger peptides (MW: 2-8 kDa). Several IAP-based methods have been developed both for plasma and urine, and they have been summarized in a review[43]. Among the peptide classes described in this review (which includes also methods for insulins and the synthetic ACTH analogue Synacthen®), detection of GHRH, LHRH and IGF-1 and analogues are described. Since MGF is a splice variant of IGF-1, they could theoretically be screened together, provided an antibody with reactivity for both peptides is used, but this has not been described so far.

4.3 Metabolism studies

Studying peptide metabolism can help improving their detectability, especially because the parent peptide usually is present in urine for a very limited time. Okano *et al*. have showed how detection of the AA 1-3 metabolite of GHRP-2 can increase the detection window for this peptide[8]. Additionally, recent MS technologies allow for untargeted full scan experiments and therefore to perform retrospective data analysis, and verify presence of metabolites in suspicious samples[12].

Since there is no possibility to perform administration studies on humans for unapproved peptides, several *in vitro/in vivo* models have been studied. A comprehensive study on metabolism of GHRPs has been published by Thomas *et al*.[46]. In this work, *in vivo* (administration in rats) and *in vitro* (incubation with human serum and recombinant amidase) models were used and then compared with the administration of GHRP-2 (the only approved drug of this class) in humans. Results yield to the discovery of two additional urinary metabolites (AA 1-3(Amide) and AA 1-6). Cox *et al*. used also serum to investigate *in vitro* metabolism of AOD9604[25]. Work from Ho *et al*. described use of homogenized horse liver to simulate the metabolism of TB-500 in the horse[31]. The use of human liver microsomes and liver and kidney S9 fraction for the metabolism of peptides has also been described recently, with results which are comparable to those obtained by Thomas *et al*. with *in vitro* models (see Chapter 8).

5 Black market as a problem of public health

The trade of performance-enhancing substances represents a sub-market of the illegal drug market, a world-wide phenomenon that involves serious legal, economic, social and health issues[47,48].

Different (in some cases, overlapping) underground markets can be identified:

- Counterfeit pharmaceutical drugs: used as low-cost generic drugs
- Recreational drugs: both soft drugs (e.g.: marijuana) and hard drugs (e.g.: heroin)
- Prescription drugs: abuse of controlled substances (e.g. depressants, stimulants)
- Doping substances

In terms of volumes and social impact[48], black market of performance-enhancing drugs may appear as a minor business and a minor social emergency. Nevertheless, it is clearly a problem of the utmost priority not only for doping control authorities.

From the perspective of the Health Authorities, abuse of doping substances from black markets poses a significant risk to the health and safety of the users. Black market products are usually manufactured with no respect for meeting Good Manufacturing Practice (GMP) requirements[49,50]. A clear example of the absence of quality controls is described in Chapter 7 of this study. These formulations lack certified quali-quantitative information, regardless of the approval of the active content for therapeutic treatment.

Even when this information is correctly indicated, side effects can still be unpredictable in case of non-approved drugs. In fact these substances did not follow a standardized approval process, as in the case of approved formulations (e.g.: morphine) that are extensively studied, before and after their authorization, so that their pharmacokinetic and toxicological profiles are generally well known. Some of the side-effects caused by the abuse of ergogenic agents are well known[51], whereas some others are still to be investigated[52]. Numerous cases have been described particularly for anabolic steroids[53–55], EPO[56,57] and blood transfusions[58]. Concerning peptide hormones active on the GH/IGF-1 axis, their link with cancer is still to debate. Theoretically, GH/IGF-1 may provide an antiapoptotic environment

that may favour survival of cancer cells, thus more studies are necessary to finally reject the risks. Continuous use of LHRH analogues can lead to hypogonadism and bone loss[59].

Though no cases related to use of black market peptides have been reported so far it is worth mentioning the peptide melanotan II. The use of this non-approved tanning agent, with no doping properties but also extremely popular on the internet markets, has been recently linked to arising of skin cancer[60].

Moreover, some of these peptides (e.g.: GHRP-2) were identified in products which were sold as "nutritional supplements",as if they were not pharmaceutical drugs[10].

One of the most alarming cases was recently described by Walpurgis *et al.*[61], who detected an 18 kDa unknown fusion protein with no correlation to doping, but supposedly a product of an empty expression vector without the DNA insert of interest used for recombinant protein production.

6 Conclusions and perspectives

Black market peptides represent a growing threat to sports that must be approached at three different levels: the illegal trade, the risks for the health, and their use to cheat in sport.

Collaboration between the different authorities has a crucial role in this battle: numerous efforts have already been put in the last years to increase awareness and to put limitations on this phenomenon, and to identify new potential doping substances, which is the first important step for monitoring the misuse in sports.

Contemporarily, public health authorities have the important role to inform and educate the athletes not only on the immorality of cheating, but also on the risks of using these unsafe formulations, similarly as what has been done for other groups of substances.

Concerning the work of doping control laboratory, the importance of preventing doping controls for these substances has been highlighted in the past[62]. As described in this

chapter, effective testing of these substances faces additional analytical issues, which are summarized in Figure 5.2.

The studies performed in the last years have provided strategies and tools to face misuse of these peptides, including general strategies for identification, detection and development of alternative models to perform PK studies. Although it t would be interesting also to to study the effects of peptides with an unclear spectrum of action for a better understanding of their effectiveness as doping agents, similarly to what has been done for steroids[63].

Figure 5.2 Summary of the issues related to doping control analysis of black market peptides.

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Chapter 6

Identification of a C-terminal amidated mechano growth factor (MGF) analogue in black market products.

Adapted from:

S. Esposito, K. Deventer, P. Van Eenoo. Characterization and identification of a C-terminal amidated mechano-growth factor (MGF) analogue in black market products. *Rapid Comm. Mass. Spectr.* **2012**, 26, 686.

1 Abstract

Mechano growth factor (MGF) is a splice variant of insulin-like growth factor that possesses anabolic properties and has not yet been approved for therapeutic use. Nevertheless, MGF is readily available on the black market. Although the World Anti-Doping Agency (WADA) banned the use of MGF in sports, no routinely performed methods have been reported for its detection. In this work, two preparations from the black market containing an unknown MGF analogue were characterized.

Mass spectrometry characterizations of unknown preparations and reference human MGF were performed on Exactive Orbitrap® and triple quadrupole mass spectrometers after liquid chromatography. High accuracy measurements allowed direct matching of experimental spectra from full scan MS with data generated *in silico*, whereas low resolution full scan MS/MS provided further information on fragmentation.

HCD scan of the analytes showed the presence of common *b* series fragments for black market preparations and human MGF reference standard, but all *y* series ions starting from $(y_1)^+$ exhibited a difference of 1 Da in nominal mass. This difference was demonstrated to be due to a C-terminal amidation of MGF. High resolution data demonstrated that the products from black market were both identified as C-terminal amidated analogues of human MGF. Additionally, low resolution MS/MS characterization revealed a potentially diagnostic transition (*m/z* 717.8 → 431.1) for the detection of C-amidated MGF and discrimination from the endogenous form.

2 Introduction

The use of peptide hormones with performance-enhancing properties is prohibited in sports. Consequently, these substances appear on the prohibited list issued by the World Anti-Doping Agency (WADA) under paragraph S2[1].

However, the availability of black market performance-enhancing products containing peptide hormones is increasing, as indicated in numerous recent publications[2–5]. Identification of these compounds is a first important step toward their detection in biological matrices[6].

One of the prohibited peptide hormones listed by WADA is human mechano-growth factor (MGF). Moreover, due to its hormonal properties MGF is prohibited by (inter)national regulations in many countries, including Belgium[7].

MGF is a 24 amino acid peptide (sequence: YQPPSTNKNTKSQRRKGSTFEERK) also known as IGF-1Ec, generated from alternative splicing of the gene for insulin-like growth factor 1 (IGF-1)[8].

Its structure, pharmacological properties, and expression of MGF-related genes have been extensively studied and described[9,10]. The potential misuse of MGF as a doping agent in sports has also been described[10–13]. Mechanical stimulation related to prolonged exercise and muscle damage up-regulates IGF-1 gene splicing, producing predominantly MGF. This MGF production has been mainly associated with anabolic processes including muscle growth and regeneration by activating muscle stem cells[9,14,15].

MGF has not yet been approved for any therapeutic use, and only preclinical studies have been reported[16,17]. Nevertheless, the bodybuilding literature on websites suggests that MGF is available as intramuscular formulations.

Although WADA has already included MGFs in the Prohibited List, there is only anecdotal evidence of the misuse of this peptide hormone. No cases of the identification of MGF in black market products have yet been reported and no doping control method for its detection has been developed.

In order to focus attention both on the potential abuse of MGF and on the risks related to the low quality of black market products, the structural characterization of an MGF analogue in two black market products by ultra-high performance liquid chromatography/high-resolution mass spectrometry (UHPLC/HRMS) and high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS) is presented here.

3 Materials and methods

3.1 Chemicals and reagents

A reference standard of the human MGF was purchased from Phoenix Pharmaceuticals, Inc. (Belmont, CA, USA) and this will be further referred to as hMGF-R.

Acetonitrile and water were purchased from BioSolve (Lexington, MA, USA). Glacial acetic acid was purchased from Merck (Darmstadt, Germany) and trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2 Unknown black market products

The first black market product containing MGF was confiscated by Belgian Customs during a routine control. The unlabeled, sealed vial contained approximately 10 mg of a white powder and this sample will be further referred to as MGF-C.

The second black market product was bought from a website. The vial contained approximately 30 mg of white powder and presented the label *'Mechano Growth Factor - IGF-13c – 2000 mcg - This product is for laboratory research purposes only. Can be harmful if used inappropriately. Keep out of reach of children*'. This substance will be referred to as MGF-W. Both vials are shown in Figure 6.1. Stock solutions from lyophilized powder at a concentration of 10 mg/mL were prepared by dissolving the powder in aqueous acetic acid (2% v/v). Before MS experiments were carried out, the samples were diluted 1:100 with water/acetonitrile/acetic acid/TFA (95:5:0.1:0.01, v/v/v/v).

Figure 6.1 Vials of MGF-C (left) and MGF-W (right).

3.3 Liquid chromatography

UHPLC separation was achieved with a Surveyor MS Pump Plus coupled with a Surveyor Plus autosampler (Thermo Scientific, Bremen, Germany) using a Poroshell 120 EC-C8 column (2.1 50mm, 2.7 mm; Agilent Technologies, Böblingen, Germany).

HPLC separation prior to low-resolution MS/MS was achieved with a Surveyor MS Pump Plus coupled with a Surveyor Plus autosampler using a Zorbax RX-C8 reverse phase column (2.1 150 mm, 5.0 mm; Agilent Technologies, Santa Clara, CA, USA). For both UHPLC and HPLC experiments 30 µL were injected for each sample. The same binary gradient was used in both sets of experiments: mobile phase A consisted of water, 0.1% acetic acid, 0.01% TFA; and mobile phase B consisted of acetonitrile, 0.1% acetic acid, 0.01% TFA. Gradient elution started from 95% A for 1.5 min, decreased linearly to 0% A in 8.5 min, and was held at 0% A for 2 min. Finally, 95% A was eluted for 3 min to equilibrate the system, for a total run time of 15 min. A constant flow rate of 200 mL/min was maintained in both cases.

3.4 High-resolution mass spectrometry

Mass spectrometry characterizations of the black market products and the reference human MGF were performed on an Exactive benchtop, Orbitrap®-based mass spectrometer (Thermo Scientific), operating in positive ion full scan MS mode and higher-energy collision-dissociation (HCD) scan mode at 50 eV. The sheath gas (N_2) pressure was set to 60 (arbitrary units), the auxiliary gas (N_2) pressure was set to 30 (arbitrary units) and the capillary temperature set to 350 °C. The capillary voltage and spray voltage were set to 30 Vand 3 kV, respectively. The instrument operated in full scan mode from m/z 100 to 2000 at 100 000

resolving power. Approximately 10 scans were averaged per spectrum. The automatic gain control (AGC) setting was 106. The data acquisition rate was 2 Hz. The Orbitrap performance was evaluated daily and, when required, external calibration was carried out with Exactive Calibration Kit solutions (from both Sigma-Aldrich and ABCR GmbH & Co. KG, Karlsruhe, Germany).

3.5 Low-resolution MS/MS

MS/MS characterization was performed on a TSQ Quantum Discovery Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with an ESI source operating in positive mode The ESI–MS operating variables used in this study were as follows: capillary voltage, 3.5 kV; source temperature, 350 °C; sheat gas (nitrogen) pressure, 30 psi; auxiliary gas (nitrogen) pressure, 10 psi; tube lens offset, 84V.

3.6 Data analysis

ProMass software from Thermo Fisher (Bremen, Germany) was used for spectral deconvolution. Protein Prospector[18] was used to compare and match acquired HCD spectra to theoretical spectra generated *in silico*.

4 Results

4.1 High resolution mass spectrometry

In order to identify the active ingredients in the black market products, solutions of the MGF-C and MGF-W samples were injected for LC/HRMS analysis, together with hMGF-R. The chromatographic behavior of the three compounds was similar, with retention times within 3.20–3.25 min. After full scan MS analysis, data deconvolution of the spectra of the three peptides provided charge state and molecular weight (MW) information on the compounds. The experimental MW of hMGF-R was 2866.4786 Da (mass error: 0.4 ppm), while the MWs of MGF-C and MGF-W were, respectively, 2865.4958 Da and 2865.4950 Da, both approximately 1 Da lower in nominal mass than the endogenous species. HCD scans of the

analytes at 50 eV, presented in Figure 6.2 and 6.3, and summarized in Table 6.1, showed the presence of several common product ions for all the three investigated peptides, including $(b_2)^+$, $(b_{21})^{++}$, $(b_{22})^{++}$, $(c_1)^+$, and the internal ion (SQRRK/KSQRR – NH_3)$^+$. On the other hand, all *y* series ions that were detected for MGF-C and MGF-W exhibited a difference in the *m/z* value that corresponds to a 1 Da nominal mass also observed in the MW. First, substitution of an amino acid was considered. *In silico generated fragmentation pathways allowed two hypotheses to explain the difference of 1 Da: Substitution of Glu_{21} or Glu_{22} with either a Gln or a Lys*. This substitution of Glu_{21} or Glu_{22} with a Gln or a Lys should lead to all the $(y)^+$, ions starting from $(y_3)^+$ or $(y_4)^+$, having a negative difference of 0.9839 or 0.9472 *m/z* units, respectively. However, the difference was observed for all the $(y)^+$ product ions. *Hence, it was concluded that a modification was already present in the* $(y_1)^+$.

Modification of the C-terminus of peptides is a common strategy in pharmaceuticals to prolong the half-life of peptide drugs[19], and amidation is frequently used for this purpose.

This modification causes a change in the nominal mass of the protein of -0.9839 Da, and the observed difference between the C-terminal Lys_{24} residue, with a theoretical m/z value of 147.1128 for human MGF and 146.1289 for its postulated amidated version, was 0.9839.

The presence of amidated Lys_{24} in the black market products was also confirmed experimentally by the presence of the product ion at m/z= 146.1288 (mass error: 0.4 ppm) in *both MGF-C and MGF-W. The fragment corresponding to the amidated* $(y_1)^+$ *was not detected in hMGF, that only exhibited the ion at m/z= 147.1129,* (mass error: 0.5 ppm). Extracted ion chromatograms in Figure 6.4 clearly illustrate these findings.

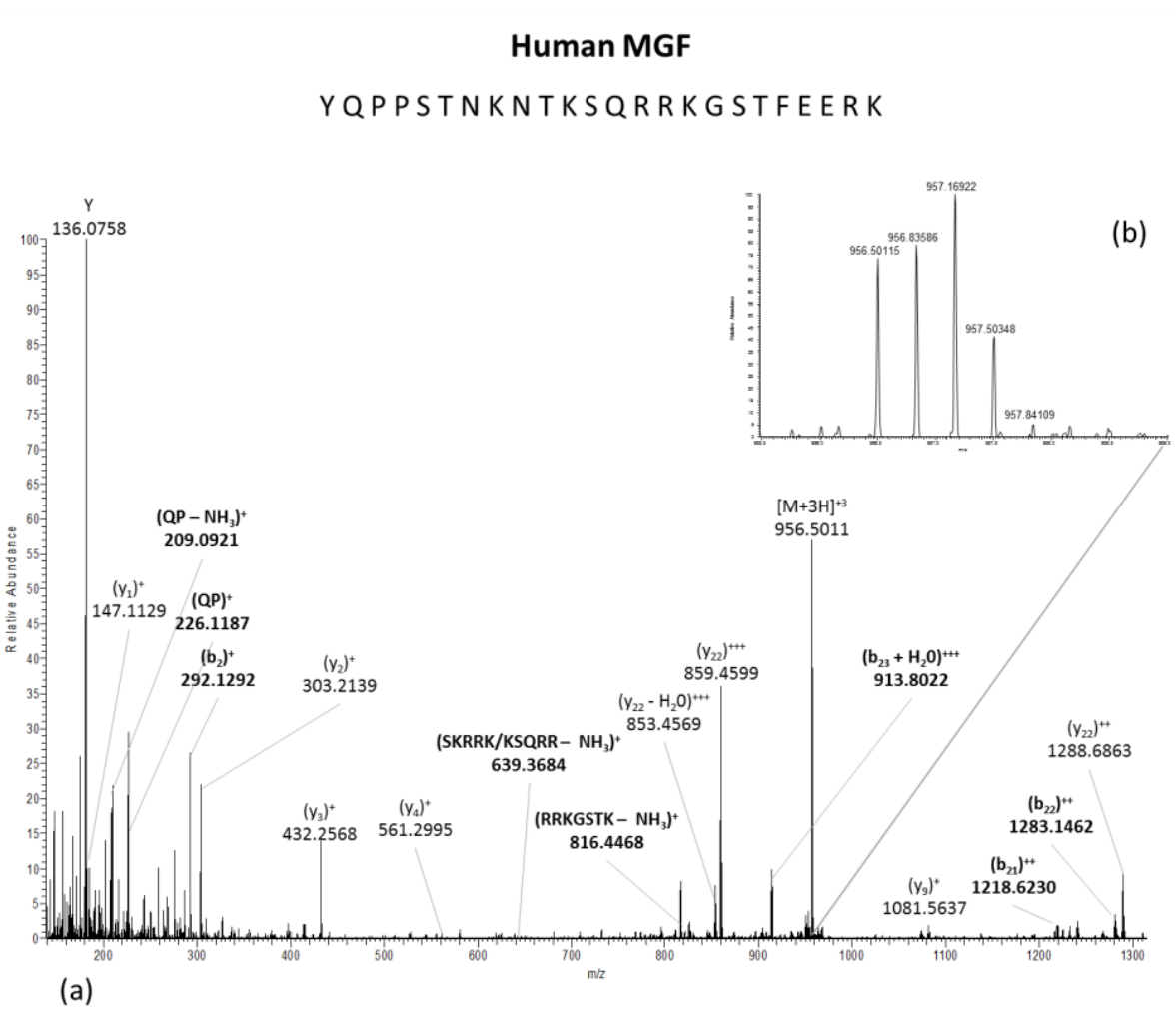

Figure 6.2 High-resolution HCD spectra of human MGF (a) and isotopic pattern of its base peak $[M + 3H]^{(3+)}$ (b), acquired at 50 eV. Product ions which are in common with those in the amidated analogue are in bold type.

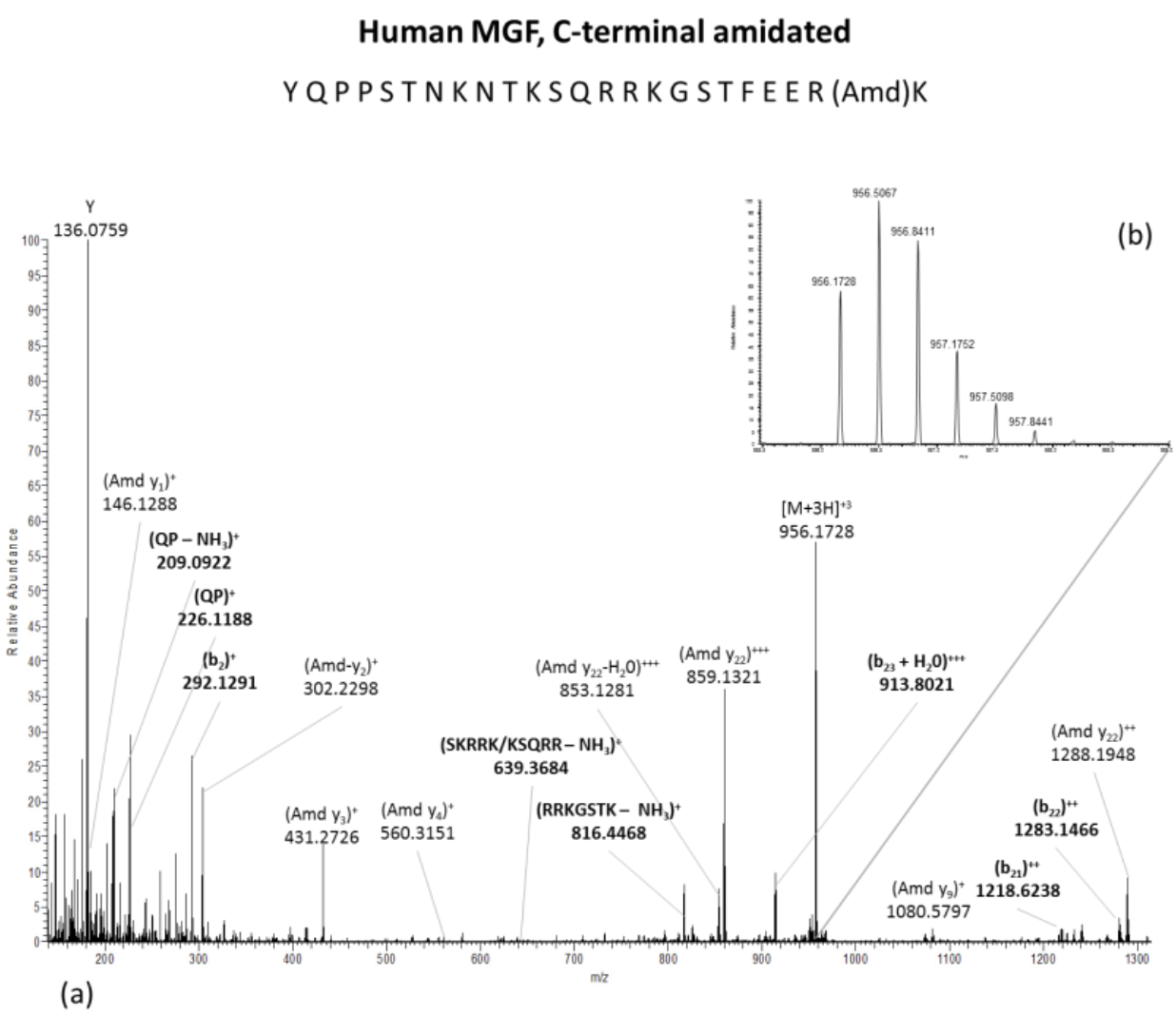

Figure 6.3 High-resolution HCD spectra of C-terminal amidated MGF (a) and isotopic pattern of its base peak [M + 3H](3+) (b), acquired at 50 eV. Common product ions are in bold type.

Table 6.1 Mass spectrometric results for the structural characterization of MGF and for the qualitative identification of C-terminal amidated MGF from black market vials after HCD scan at 50 eV

Product ion type		Amino acid sequence	Theoretical *m/z*	Detected in	Experimental *m/z*	Accuracy (Δppm)
Common product ions	$(b_2)^+$	YQ	292.1292	hMGF-R	292.1292	0.1
				MGF-C	292.1291	0.2
				MGF-W	292.1292	0.1
	Internal fragment	SKRRK/KSQRR – NH_3	639.3687	hMGF-R	639.3684	0.3
				MGF-C	639.,3684	0.2
				MGF-W	639.3689	0.5
	$(b_{21})^{++}$	YQPPSTNKNTKSQRRKGSTFE	1218.6226	hMGF-R	1218.6230	0.3
				MGF-C	1218.6238	1.0
				MGF-W	1218.6229	0.3
	$(b_{22})^{++}$	YQPPSTNKNTKSQRRKGSTFEE	1283.1440	hMGF-R	1283.1462	1.8
				MGF-C	1283.1466	2.0
				MGF-W	1283.1444	0.4
	$(c_1)^+$	Y	181.0972	hMGF-R	181.0973	0.2
				MGF-C	181.0972	0.1
				MGF-W	181.0972	0.4
Discriminating product ions	$(y_1)^+$	K	147.1128	hMGF-R	147.1129	0.5
		K(Amd)	146.1289	MGF-C	146.1288	0.4
				MGF-W	146.1288	0.3
	$(y_2)^+$	RK	303.2140	hMGF-R	303.2139	0.1
		RK(Amd)	302.2300	MGF-C	302.2298	0.4
				MGF-W	302.2298	0.5
	$(y_3)^+$	ERK	432.2567	hMGF-R	432.2568	0.3
		ERK(Amd)	431.2725	MGF-C	431.2726	0.3
				MGF-W	431.2727	0.5
	$(y_{22})^{+++}$	PPSTNKNTKSQRRKGSTFEERK	859.4600	hMGF-R	859.4599	0.1
		PPSTNKNTKSQRRKGSTFEERK(Amd)	859.1320	MGF-C	859.1321	0.1
				MGF-W	859.1323	0.3
	$(y_9)^+$	KGSTFEERK	1081.5636	hMGF-R	1081.5637	0.1
		KGSTFEERK(Amd)	1080.57960	MGF-C	1080.5797	0.1
				MGF-W	1080.5799	0.3

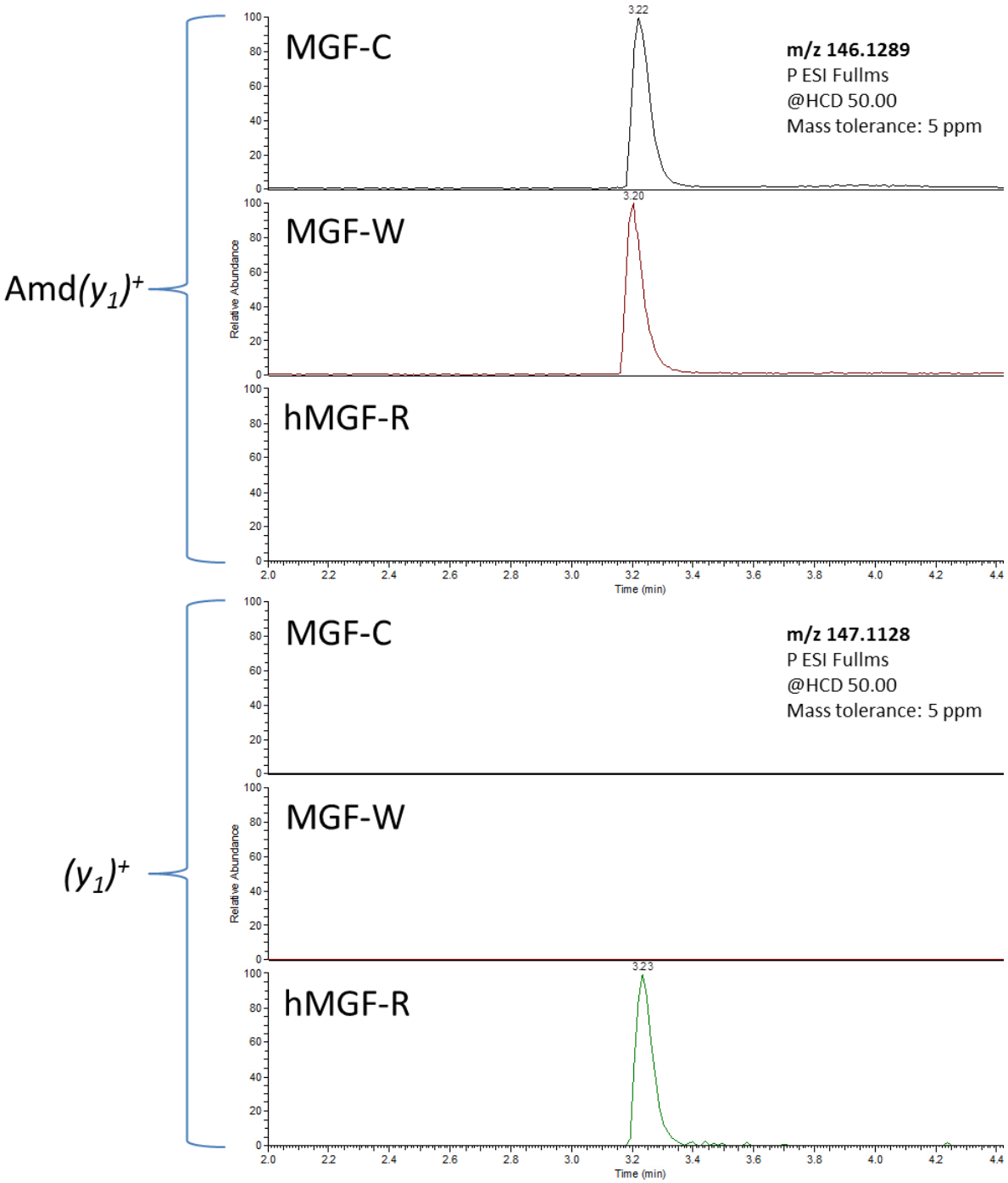

Figure 6.4 Extracted ion chromatograms for the theoretical product ions $(Amd\ y_1)^+$, *m/z* 146.1288, and $(y_1)^+$, *m/z* 147.1129, corresponding, respectively, to the amidated and non-amidated C-terminal lysine. $(Amd\ y_1)^+$ was detected both in MGF-C and MGF-W, whereas $(y_1)^+$ was detected only in hMGF-R.

4.2 Low-resolution MS/MS

Figure 6.5 shows MS/MS product ion spectra of hMGF (Figure 6.5a) and MGF-C (Figure 6.5b) from the precursor ion 717.8 ($[M+4H]^{+4}$) recorded at 30 eV. The MGF-W spectrum is not presented, since it is identical to the one of MGF-C. Both spectra present several high abundant *b* and *c* product ions with the same nominal *m/z* values. Product ions corresponding to internal fragments or single aminoacids were also present. Three *y* ions of significant abundance were recorded: $(y_3)^+$, $(y_{22})^{3+}$, and $(y_{22}\text{-}H_2O)^{3+}$. Among these, only the singly charged $(y_3)^+$ ion exhibited a nominal difference of 1 *m/z* unit (*m/z* 432.2 for hMGF, *m/z* 431.1 for the C-amidated analogue) which allows a discrimination between the two forms. The extracted ion chromatogram (Figure 6.5c) shows that this product ion was detected only in the black market products.

Therefore, the transition 717.8→ 431.1 can discriminate the C-amidated form from the endogenous using low resolution tandem mass spectrometry and, potentially, be used to detect MGF misuse in biological fluids.

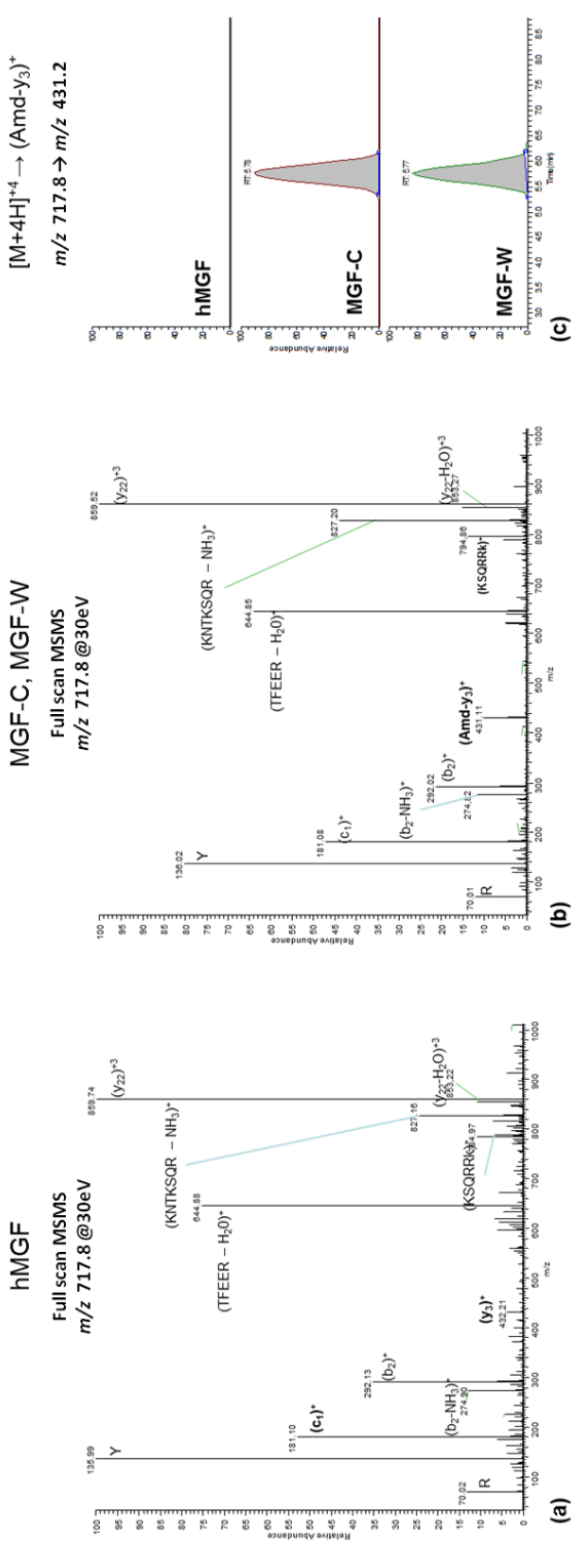

Figure 6.5 Low-resolution full scan MS/MS spectra of product ions of hMGF (a) and MGF-W (b) from precursor ion *m/z* 717.8. MGF-C, not shown for brevity, as its spectrum is identical to that of MGF-W. The product ions $(y_3)^+$, and (*Amd* y_3)+ show a difference of 1 *m/z* unit and can be used to discriminate the two forms, as shown in the extracted ion chromatogram (c).

4.3 Detection of other ingredients

The contents of the vials were also analyzed in order to detect anabolic steroids, and stimulants, using previously described methods[20–22]. None of these substances were detected. During full scan HR-MS analysis, mannitol was detected. Mannitol is an osmodiuretic agent also prohibited by WADA[23]. However, the low amount of mannitol present in the vials should not induce a significant diuretic effect and its presence is more likely to be related to its assistance during the lyophilization of the peptides[24].

5 Discussion

Unlabeled, fake and/or wrong labeled vials are generally observed for black market products[2,25,26].

The MGF-C vial did not have any labeling at all. The MGF-W vial exhibited a gross error on its label (*IGF-13c* instead of *IGF-1Ec*) and the amidation at the C-terminus is not mentioned. Hence, the difference between the declared active compound and the real compound present in the black market product can have unpredictable consequences on biological and toxicological effects as well as on the detectability[26]. Hence, the described products are clearly illegal, both from a commercial and ethical point of view, but they also present a great risk for the health and safety of the consumers.

In particular, these products (of doubtful quality) allow prices which are extremely low when compared with those from pharmaceutical companies. These prices make them very attractive for the customers.

The seized vials are sold for intravenous, intramuscular or subcutaneous injections which may cause infections when not sterile. As mentioned in the Introduction, the biggest risk associated with human MGF is that it is not an approved drug and, in particular, the C-terminal amidated version of MGF is not even described in the literature as a therapeutic candidate. Therefore, there are no toxicological studies, clinical trials or standardized protocols for the therapeutical use of the product in the black market vials. In most cases, internet suppliers are aware of these problems and to avoid claims they sell them frequently

with the advice *'intended for laboratory and research use only*'. Although the products are officially sold as a research chemical and not for human consumption, the websites selling the products leave no doubt as to the intended purpose. Indeed, in some cases free syringes and bacteriostatic water are provided with the products. In the context of doping control analysis, these black market products cause great difficulties in their detection. In fact, small changes in the structure of a peptide (e.g. amidation of MGF) can have negative effects on their detectability, which is mostly based on mass spectrometric detection.

6 Conclusions

A C-terminal amidated analogue of human MGF was successfully identified in two black market products by means of LC/HRMS. This report demonstrates that further attention must be paid to such MGF preparations, in order to evaluate realistically its misuse in sport as a doping agent and the potential health dangers associated with low-quality manufactured black market products. Knowledge of the available products and their contents is required to develop efficient strategies for the detection of human MGF and its analogues in biological fluids doping control purposes.

7 Acknowledgements

The Laboratory of Customs and Excises (Belgium) is gratefully acknowledged.

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Chapter 7

Identification of the growth hormone-releasing hormone analogue [Pro1, Val14]-hGHRH with an incomplete C-term amidation in a confiscated product

Adapted from:

S. Esposito, K. Deventer, P. Van Eenoo. Identification of the growth hormone-releasing hormone analogue [Pro1, Val14]-hGHRH with an incomplete C-term amidation in a confiscated product *Drug Test. Anal.* **2014**, DOI: 10.1002/dta.1730

1 Abstract

In this work, a modified version of the 44 amino acid human growth hormone-releasing hormone (hGHRH(1-44)) containing an N-terminal proline extension, a valine residue in position 14, and a C-terminus amidation (sequence: PYADAIFTNSYRKVVLGQLSARKLLQDIMSRQQGESNQERGARARL-NH_2) has been identified in a confiscated product by liquid chromatography-high resolution mass spectrometry (LC-HRMS). Investigation of the product suggests also an incomplete C-term amidation.

Similarly to other hGHRH analogues, available in black markets, this peptide can potentially be used as performance-enhancing drug due to its growth hormone releasing activity and therefore it should be considered as a prohibited substance in sport. Additionally, the presence of partially amidated molecule reveals the poor pharmaceutical quality of the preparation, an aspect which represents a big concern for public health as well.

2 Introduction

The use of peptide-based doping agents has become increasingly popular during the last few years[1]. A relevant phenomenon for this class of substances is represented by unapproved peptide formulations[2–6]. According to the Prohibited List of substances and methods of the World Anti-Doping Agency (WADA)[7], any pharmacological substance without current approval by any governmental regulatory health authority for human therapeutic use, is prohibited.

Unapproved drugs are generally not accessible through legal channels of distributions, but several cases have already demonstrated how they can find their way into black markets[2–6,8,9]. This phenomenon represents a big concern for public health, since these preparations are often prepared with no respect for good manufacturing practice, with lack or even total absence of information on composition. Moreover, these peptides do not usually undergo a complete toxicological evaluation (e.g.: TB-500)[6], and therefore the risks associated with the intake are unpredictable.

In sport drug testing, identification of new peptides with performing-enhancing properties is a first important step towards the development of effective detection methods for monitoring their misuse in sports[10,11]. In this context, collaboration between controlling authorities and laboratories has a crucial role in the battles against illegal trade of peptide hormones.

Recently, an unknown preparation, consisting of an unlabeled vial containing lyophilized powder (Figure 7.1), was confiscated by Belgian Customs authorities and then delivered to our laboratory for identification. The product was characterized by ultra-high performance liquid chromatography/high-resolution mass spectrometry (UHPLC-HRMS).

Figure 7.1 The product confiscated by Belgian Customs.

3 Materials and methods

3.1 Chemicals and reagents

Acetonitrile and water (LC grade) were purchased from BioSolve (Lexington MA, USA). Glacial acetic acid was purchased from Merck (Darmstadt, Germany). Formic acid was purchased from Fischer Scientific (Loughborough, UK). Sequencing Grade Modified Trypsin was purchased from Promega (Madison, WI, USA). Ammonium bicarbonate was purchased by Sigma-Aldrich (St. Louis, MI, USA)

Stock solution of the unknown product was prepared by dissolving the content of the vial in water (1 mg/mL). For direct injection, sample was diluted 1:10 with 2% acetic acid.

3.2 Trypsin digestion

100 µL stock solution was evaporated to dryness (40 °C) and reconstituted in 200 µL trypsin solution (10 µg/mL in 25mM ammonium bicarbonate buffer, pH 8). Sample was incubated under gentle stirring for 24 hours at 37 °C. Trypsin digestion was stopped with 2% acetic acid (50 µL), and samples were then injected in the LC-MS system.

3.3 Liquid chromatography

The UHPLC system consisted of an Accela LC (Thermo Scientific, Bremen, Germany) equipped with degasser, Accela 1250 pump, autosampler thermostated at 10°C and a heated column compartment. LC separation was performed using a Zorbax SB-C8, 2.1 x 50 mm and 1.8 µm particle size from Agilent Technologies (Böblingen, *Germany*) thermostated at 25°C. The mobile phases were 0.2% formic acid in water (A) and 0.2% formic in acetonitrile (B). The gradient program was as follows: 100% A for 5 min, then decreased linearly to 0% in 8.0 min, and held at 0% for 2 min followed by an increase to the initial concentration of 100% A in 0.1 min. Equilibrium time was 5 min resulting in a total run time of 20 min. The flow rate was set constant at 250 µL/min and the injection volume was 10 µL.

3.4 High-resolution mass spectrometry

HRMS characterization was performed using a Q-Exactive benchtop, Orbitrap™-based mass spectrometer (Thermo Scientific, Bremen, Germany) operated in positive-negative polarity switching mode and equipped with a heated electrospray ion source (HESI). Nitrogen sheath gas flow rate and auxiliary gas were set at 60 and 30 (arbitrary units), respectively. The capillary temperature was 350°C, the spray voltage 3 kV or -3 kV and the capillary voltage 30 V or -25 V, respectively in positive or negative ion modes. The instrument operated from *m/z* 300 to 3 000 at 70 000 resolving power in full scan (FSMS). The automatic gain control (AGC) was set to 10e6. The data acquisition rate was 2 Hz.

FSMS experiments were followed by targeted MS/MS (tMSMS) experiments at a normalized collision energy (NCE) set at 30% for peptide sequencing. For tMSMS experiments, the instrument operated from *m/z* 100 to 1 500 at 17 500 resolving power and AGC set to 10e6. Isolation window of the quadrupole was set to 3.0 *m/z.* The Orbitrap™ performances in both positive and negative ionization modes were evaluated daily and when it failed, external calibration was performed with Exactive Calibration Kit solutions (Sigma-Aldrich, and ABCR GmbH & Co. KG, Karlsruhe, Germany). A mass extraction window of 5 ppm was used.

3.5 Data analysis

ProMass software from Thermo Fisher (*Bremen, Germany*) was used for spectral deconvolution. Protein Prospector v 5.10.19 was used to predict both trypsin digestion products and fragmentation of peptides for de novo sequencing[12].

4 Results

4.1 Identification of [Pro1, Val14]-hGHRH

HRMS is an ideal tool for identification of peptidic compounds due to the possibility to determine the amino acid sequence of a peptide based upon its MS/MS spectrum. Preliminary analysis consisted of direct injection for FSMS analysis. Figure 7.2 shows full scan FSMS spectrum of the unknown compound, which presents a cluster of multicharged ions in positive mode (typical for peptides) in the *m/z* range 500-2000, whereby the six-fold charged ion at *m/z* 872.9687 is the most abundant. Mass spectrum deconvolution yielded a monoisotopic mass 5233.7555 Da.

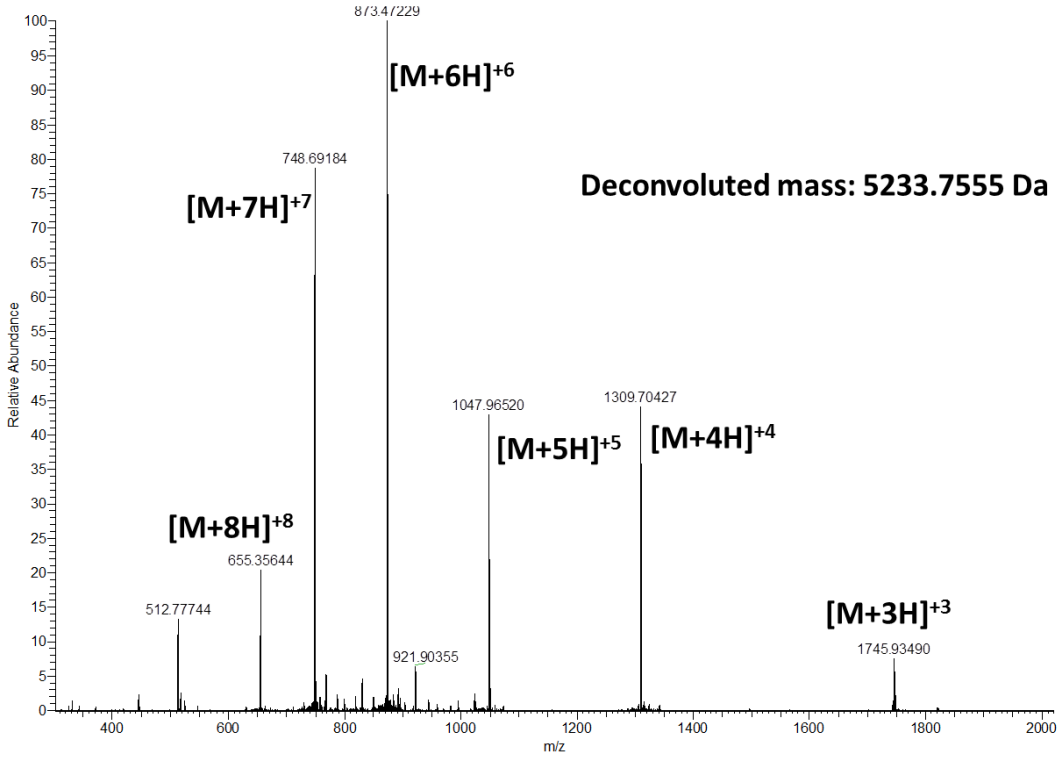

Figure 7.2 Full scan spectrum of the unknown peptide.

In order to obtain better peptide sequencing, the peptide was incubated with the proteolytic enzyme trypsin. Tryptic digestion allowed for the formation of smaller subunits of the peptide (MW < 2000 Da) which could be easily sequenced for the identification of the intact peptide. Tryptic product peptides that were identified are all summarized on Table 7.1. As shown in Figure 7.3 for six representative peptides, de novo sequencing from MS/MS spectra of tryptic products generated by enzymatic digestion of the native peptide provided identification of the unknown substance as a 46-AA modified version of human GHRH(1-44, corresponding to the sequence PYADAIFTNSYRKVVLGQLSARKLLQDIMSRQQGESNQERGARARL and to an exact monoisotopic mass of 5233,7637 Da, confirming the value obtained from spectral deconvolution of the native peptide. The peptide has a proline residue at the N–terminus (Figure 7.3a and 7.3b), a valine residue in position 14 (Figure 7.3b and 7.3c) and a C-term amidation which are not present in hGHRH and it will be further referred as [Pro1, Val14]-hGHRH.

The valine in position 14 was the most surprising finding, since it was inserted as an additional amino acid and not, as expected, in substitution of an amino acid (perhaps arginine or lysine, sensible to enzymatic degradation). On the other hand, N-terminal proline extension and C-terminal amidation have been described for GHRH analogues[13,14].

Cleavage sites for [Pro1, Val14]-hGHRH were the carboxyl side of $Arg_{12,22,31,39,43}$, and $Lys_{13,23,}$ which are typical cleavage sites for trypsin. Digestion products with missing cleavages were also detected (due to incomplete digestion); however, these peptides provided further confirmation of the sequence.

Table 7.1 List of tryptic peptides detected after digestion, including those with missing cleavages.

Theoretical *m/z*	Experimental *m/z*	Accuracy (Δppm)	Charge	Start-End	Sequence
872.9654	872.9687	+2.3	6+	1-44	Native (free)
872.8137	872.8153	+2.1	6+	1-44	Native (amidated)
359.2401	359.2394	-1.9	+	44-46	ARL (free)
358.2561	358.2554	-1.9	+	44-46	ARL (amidated)
471.7902	471.7896	-1.2	++	14-22	VVLGQLSAR
488.2682	488.2674	-1.6	++	24-31	LLQDIMSR
535.8377	535.8367	-1.2	++	14-23	VVLGQLSARK
552.3157	552.3145	-2.1	++	23-31	KLLQDIMSR
560.3132	560.3122	-1.7	++	23-31	KLLQDIM(so)SR
567.2909	567.2901	-1.4	+++	32-46	QQGESNQERGARARL (free)
678.0007	677.9998	-1.3	+++	24-40	LLQDIMSRQQGESNQER
709.3410	709.3406	-0.3	++	1-12	PYADAIFTNSYR
773.3884	773.3877	-1.1	++	1-13	PYADAIFTNSYRK
823.7798	823.7791	-0.9	+++	1-22	PYADAIFTNSYRKVVLGQLSAR

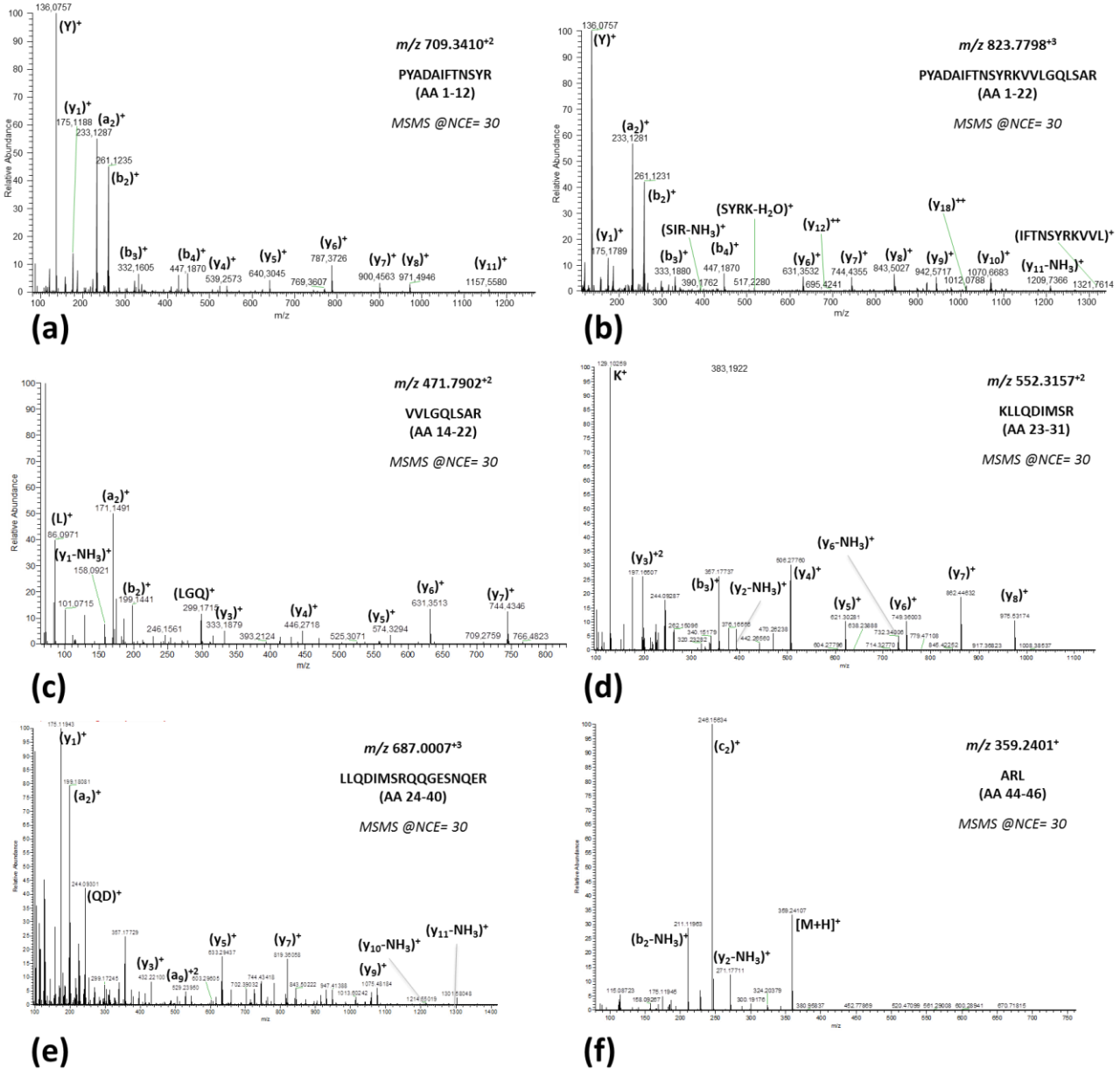

Figure 7.3 MS/MS spectra for six representative [Pro1, Val14]-hGHRH tryptic peptides.(b-f). The C-terminus peptide ARL was detected mainly in the free form (see Figure 7.5).

4.2 C-terminus: an incomplete amidation?

Analysis of the tryptic products, particularly of the product AA 44-46, revealed the presence of both free and amidated C-terminus. As shown in Figure 7.4, beside the ARL-OH pseudomolecular ion (*m/z*=359.2394) (Figure 7.4a), the ion 358.2554 (Δ*m/z*= -0.9840) (Figure 7.4b) corresponding to the amidated peptide ARL-NH_2 (theoretical *m/z*=358.2561,

Δppm= -1.9) was detected. This result was confirmed by the detection of the two $(y_2\text{-}NH_3)^+$ ions at *m/*z= 270.1922 (Δppm= -1.1) (Figure 7.4c) *m/*z=271.1758 Δppm= -2.5) (Figure 7.4d). The ratio between the areas of the peaks indicates that the free peptide is significantly more abundant than the amidated. Unfortunately, no appropriate reference standards were available to provide a more precise determination.

As trypsin does not have deamidating activity, the free form could not be generated during the digestion. Traces of the amidated form (monoisotopic mass= 5232.7560) were also detected in the native peptide (Figure 7.4e and 7.4f).

Amidation, a common strategy to improve the pharmacokinetics properties of a peptidic drug, is generally performed in the last stages of the synthesis. Moreover, amidation is present in other GHRH analogues[4]. This finding suggests an incomplete amidation of the peptide during the manufacturing process. This is a clear example of the lack of quality controls typically associated to the production of black market peptides.

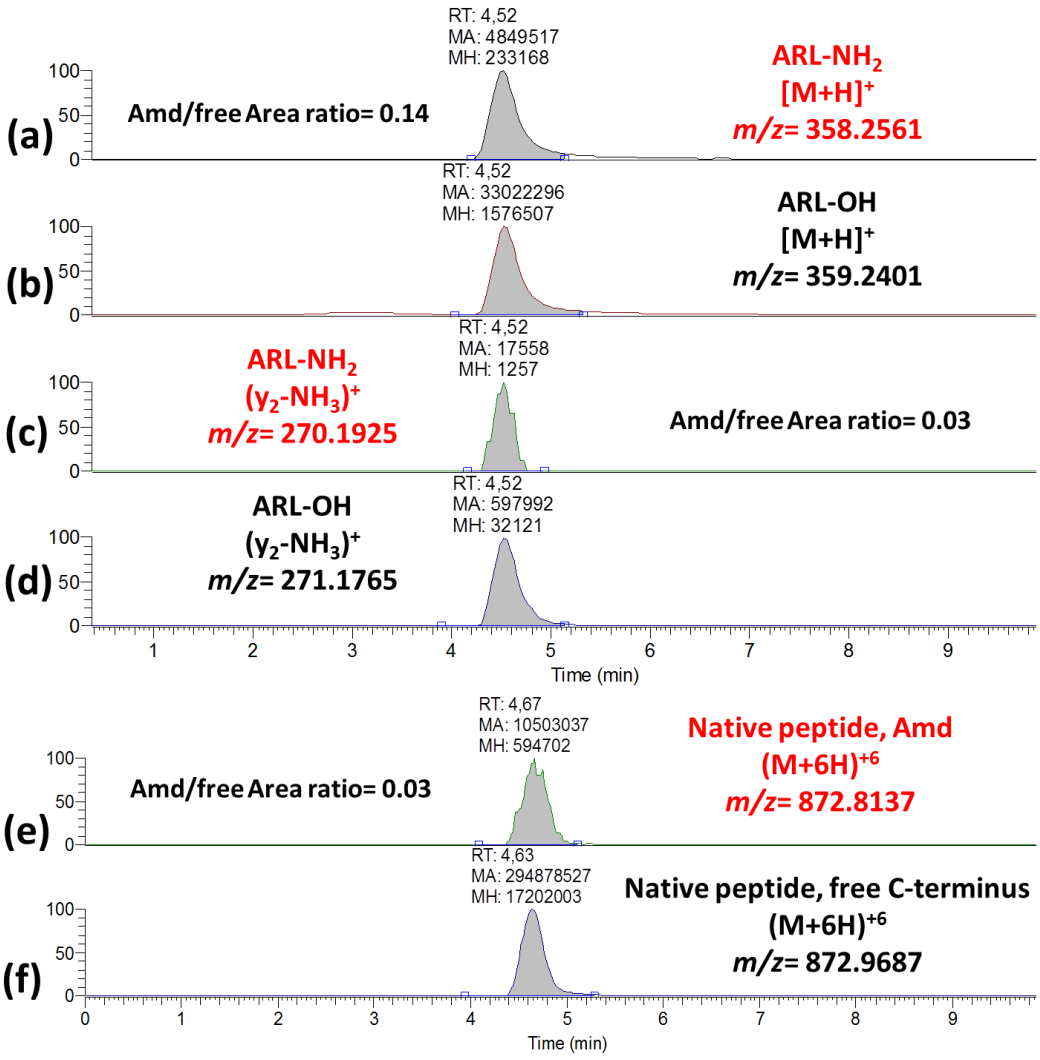

Figure 7.4 Extracted ion chromatograms and spectra of $[M+H]^+$ and $(y_2\text{-}NH_3)^+$ ions from tMSMS of tryptic product AA 44-46 ARL-OH (precursor ion at *m/z*= 359.2401) (a,c) reveal the presence, in a minor amount, of 44-46 ARL-NH_2 (b,d), most likely due to an low yield of the amidation during the synthesis of the peptide. Traces of the amidated peptides were also detected in the full scan spectrum of the native peptide (e,f).

4.3 Misuse and detection of [Pro1, Val14]-hGHRH

The human growth hormone-releasing hormone (GHRH) is a 44 amino acid hypothalamic peptide (sequence: YADAIFNTSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL) that stimulates GH production and release[14]. GHRH analogues are considered as prohibited substances according to section S2 of WADA List (peptide hormones, growth factors and

related substances)[7]. To the best of our knowledge, [Pro1, Val14]-hGHRH-NH_2 has not been approved for any clinical application.

There is reason to believe that [Pro1, Val14]-hGHRH might be marketed as performance-enhancing drug. Indeed, similar to other several other analogues of GHRH(1-44) and GHRH(1-29) (tesamorelin, sermorelin, CJC-1293 and CJC-1295)[4], [Pro1,Val14]-hGHRH appears to find its way to black market and is classified by the marketers as a performance-enhancing drug. GHRH analogues appear to be attractive especially to some communities such as bodybuilding and fitness. Obviously, no pharmacokinetics data are available for [Pro1, Val14]-hGHRH, but very low urinary and plasmatic concentrations (sub ng/mL) and short half-life can be expected. However, due to its similarity to other GHRH analogues, currently existing methods based on immunoaffinity purification followed by LC-MS detection should theoretically be able to monitor its abuse[11].

5 Acknowledgments

The Laboratory of Customs and Excises (Belgium) is gratefully acknowledged.

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Chapter 8

Identification TB-500 in a black market product, *in vitro* metabolism and synthesis of reference standard material

Adapted from:

S. Esposito, K. Deventer, J. Goeman, J. Van der Eycken, P. Van Eenoo. Synthesis and characterization of the N-terminal acetylated 17-23 fragment of thymosin beta 4 identified in TB-500, a product suspected to possess doping potential. *Drug Test. Anal.* **2012**, 4 (9), 733-8.

1 Abstract

The product TB-500, which is suspected to be used as doping agent both in equestrian, canine and human sports, was confiscated by the Belgian Customs during a routine control. The composition of the formulation was not well defined, and therefore, in a first stage, a qualitative identification was needed in order to provide information for the development of analytical methods that can be used for monitoring TB-500 misuse.

This work describes the detection and the identification of the active content of TB-500 by means of high-performance liquid chromatography/high resolution mass spectrometry using an Orbitrap Exactive benchtop mass spectrometer through a bottom-up approach.

The N-terminal acetylated 17-23 fragment of human thymosin beta 4 (sequence: Ac-LKKTETQ) was detected in the formulation. This peptide corresponds to the actin-binding domain of the human thymosin beta 4, which is known for promoting on tissue repair and angiogenesis. To unequivocally confirm the structure of the peptide, Ac-LKKTETQ was also synthesized by solid-phase peptide synthesis. The peptide was also analyzed by low resolution full scan MS/MS, in order to provide useful information on fragmentation behavior of the peptide.

Additionally, *in vitro* metabolism studies were performed using human liver microsomes and S9 fraction in order to determine the most significant metabolites, which were synthesized as well.

2 Introduction

The use of performance-enhancing peptides from black markets is a growing issue in human[1–4] as well as equine sports drug testing[5].

A product called TB-500, claimed to increase muscle growth and tissue repair in horses and other mammals, is available on the internet and officially distributed. It is presented as "the synthetic peptide of the *active* region of thymosin beta 4 (Tβ4)", without any further qualitative description such as amino acid sequence or molecular weight. Tβ4, a 43 amino acid peptide, is the most abundant member of beta thymosins[6]. Its primary function is to stimulate the production of T cells, which are an important part of the immune system. Additionally, Tβ4 has been shown to prevent apoptosis, promote cell survival, angiogenesis, and tissue regeneration in mice and rats[7].

TB-500 is intended for veterinary use only (10 mg/dose), but it is legitimate to suspect its use as doping agent not only in horse and greyhound racing, but even in humans. Indeed, TB-500 packages, containing 6 powder vials and physiological saline solutions for injection, were already confiscated by officials to persons who declared that the product was for personal use. In at least one case this received also world-wide attention from the media since it was connected to a former professional athlete which was still employed by his team[8], while in another case, a former team doctor was arrested in the possession of the substance[9]. These events suggest that TB-500 might also be used by athletes for doping purposes, although no evidence for a misuse has been reported so far.

A preventive and proactive approach against these novel formulations containing peptidic drugs without any clinical approval is mandatory, including identification of the active content of these products, in order to monitor their misuse. Additionally, methods need to be developed for their detection and preferably, well characterized reference standards need to be synthetized. In this work, the active content of TB-500 was identified and synthesized. Moreover, three TB-500 metabolites were synthesized after *in vitro* experiments.

3 Materials and methods

3.1 Chemicals and reagents

All the reagents were analytical or HPLC grade. Reference standard of human Tβ4 was purchased from Bachem (Weil am Rhein, Germany).

Acetonitrile, water, dichloromethane were purchased from BioSolve (Lexington MA, USA). Glacial acetic acid was purchased from Merck (Darmstadt, Germany); Methanol, formic acid, N,N'-diisopropylcarbodiimide (DIC), 4-dimethylaminopyridine (DMAP), N,N-diisopropylethylamine (DIPEA), piperidine, pyridine, (methyl-t-butyl ether (MTBE), and acetic anhydride were purchased from Sigma-Aldrich (S. Louis, USA).

All the fluorenylmethyloxycarbonyl chloride (Fmoc) protected amino acids were L-isomers. Fmoc-Lys(Boc)OH, Fmoc-Thr(tBu)OH, Fmoc-Glu(tBu)OH were purchased from Nova Biochem (San Diego, CA, USA). Fmoc-Leu-OH precoated WANG-Resin was purchased from Bachem. Fmoc-Gln(Trt)OH and O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) from Iris Biotech GnBH (Marktredwitz, Germany).

3.2 TB-500

A TB-500 package, confiscated by Belgian Customs during a routine control, was delivered to the laboratory (Figure 8.1). The sealed vial contained approximately 10 mg of a white powder. Stock (1 mg/mL) and working solutions (100 µg/mL and 100 ng/mL) of TB-500 and human Tβ4 from lyophilized powders were prepared by dissolving them in aqueous acetic acid (2% v/v).

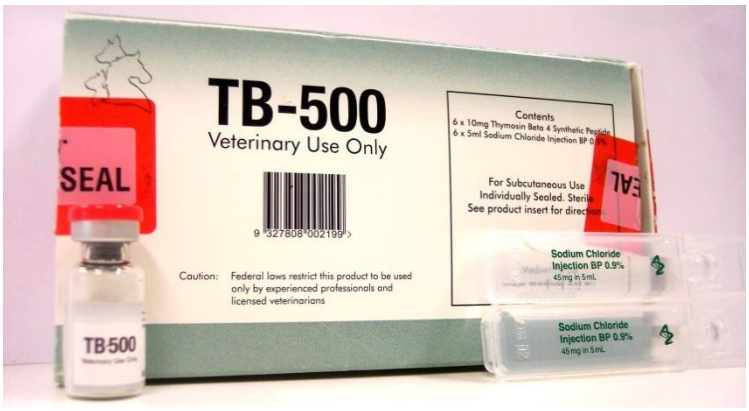

Figure 8.1 The package confiscated by the Customs containing six vials of TB-500 and saline solutions for injection.

3.3 Liquid chromatography

Chromatographic separation was achieved with a Surveyor MS Pump Plus coupled with a Surveyor Plus autosampler (Thermo Scientific, Bremen, Germany) using a Zorbax RX-C8 reverse-phase column (2.1 x 150 mm, 5 µm) from Agilent Technologies (Santa Clara, USA). The conditions were the same for both high-resolution and low-resolution MS experiments. For each sample, 5 µL were injected. A binary gradient was used: mobile phase A consisted of water containing 0.2% formic acid; mobile phase B consisted of acetonitrile containing 0.2% formic acid. Gradient elution was as follows: it started from 100% A for 5 minutes, then decreased linearly to 0% A in 10 minutes, and held at 0% A for 2 minutes. Finally, 95% A was eluted for 3 minutes to equilibrate the system for further injection experiments, for a total run time of 20 minutes. A constant flow rate of 300 µl/min was maintained.

3.4 Mass spectrometry

High-resolution mass spectrometry (HRMS) experiments were performed on an Exactive benchtop, Orbitrap-based mass spectrometer (Thermo Scientific, Bremen, Germany). The instrument operated both in positive full scan MS mode and positive higher-energy collision dissociation (*HCD)* "all ion fragmentation" MS/MS mode at 25 *eV*. The sheath gas (nitrogen) pressure was set to 60 (arbitrary units), the auxiliary gas (nitrogen) pressure set to 30 (arbitrary units) and the capillary temperature set to 350°C. The capillary voltage and spray voltage were set to 30 V and 3 kV, respectively. The instrument operated in full scan mode

from *m/z* 100–2000 at 100,000 resolving power. The data acquisition rate was 1 Hz. Approximately 10 scans were averaged per spectrum.

Low-resolution MS/MS characterization of TB-500 was performed on a TSQ Quantum Discovery Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with an ESI source operating in positive mode The ESI–MS operating variables used in this study were as follows: capillary voltage, 3.5 kV; source temperature, 350 °C; sheath gas (nitrogen) pressure, 30 psi; auxiliary gas (nitrogen) pressure, 10 psi; tube lens offset, 84V.

3.5 Data analysis

ProMass software from Thermo Fisher (*Bremen, Germany*) was used for spectral deconvolution. Protein Prospector[10] was used to compare and match acquired HCD spectra to those predicted *in silico*. Mass tolerance was set at 5 ppm.

3.6 *In vitro* metabolism

TB-500 was incubated both with human liver microsomes (HLM) and liver S9 fraction. These experiments are extensively described in Chapter 9.

3.7 Synthesis of TB-500 and metabolites

Ac-Tβ4(17-23) and its metabolites were synthesized using a standard Fmoc-SPS protocol[11].

4 Results

4.1 HRMS identification of the active content of TB-500

Solutions of TB-500 were first injected for HPLC-HRMS analysis in order to identify the active ingredient(s) in the formulation. Total ion chromatogram showed one main peak, which could be assigned to originate from a peptide-like structure at t_r= 9.1 min. The full scan MS spectrum (data not shown) revealed the presence of two molecular ions of the same molecule, respectively 445.2532 ($[M+2H]^{2+}$) and 889.4988 ($[M+H]^{+}$). Spectrum deconvolution provided a molecular weight (MW) of 888.4910. It was immediately evident that TB-500 did

not contain the endogenous Tβ4 (MW: 4963.4642). Therefore, elucidation of the structure of the detected species was accomplished by *de novo* sequencing based on HCD of the peptide precursor at 25 eV. b^+ and y^+ ions allowed *de novo* sequencing of the peptide through direct matching with theoretical data. As shown in Figure 8.2a and summarized in Table 8.1, a heptapeptide was detected, corresponding to the 17-23 fragment of human Tβ4 (sequence: LKKTETQ). Additionally, all b^+ ions showed a positive *m/z* shift of 42.0105 with respect to the Tβ4 fragment, indicating the presence of an acetylated N-terminus. The identified peptide (theoretical MW 888.4911) will be further referred to as Ac-Tβ4(17-23). Finally, a confirmation of the structure and the stereochemistry was given after synthesis of the "all L-amino acid" peptide, as shown by the HCD spectrum depicted in Figure 8.2c, by comparison of respective NMR spectra in Figure 8.2b and 8.2d, and the chromatograms in Figure 8.3.

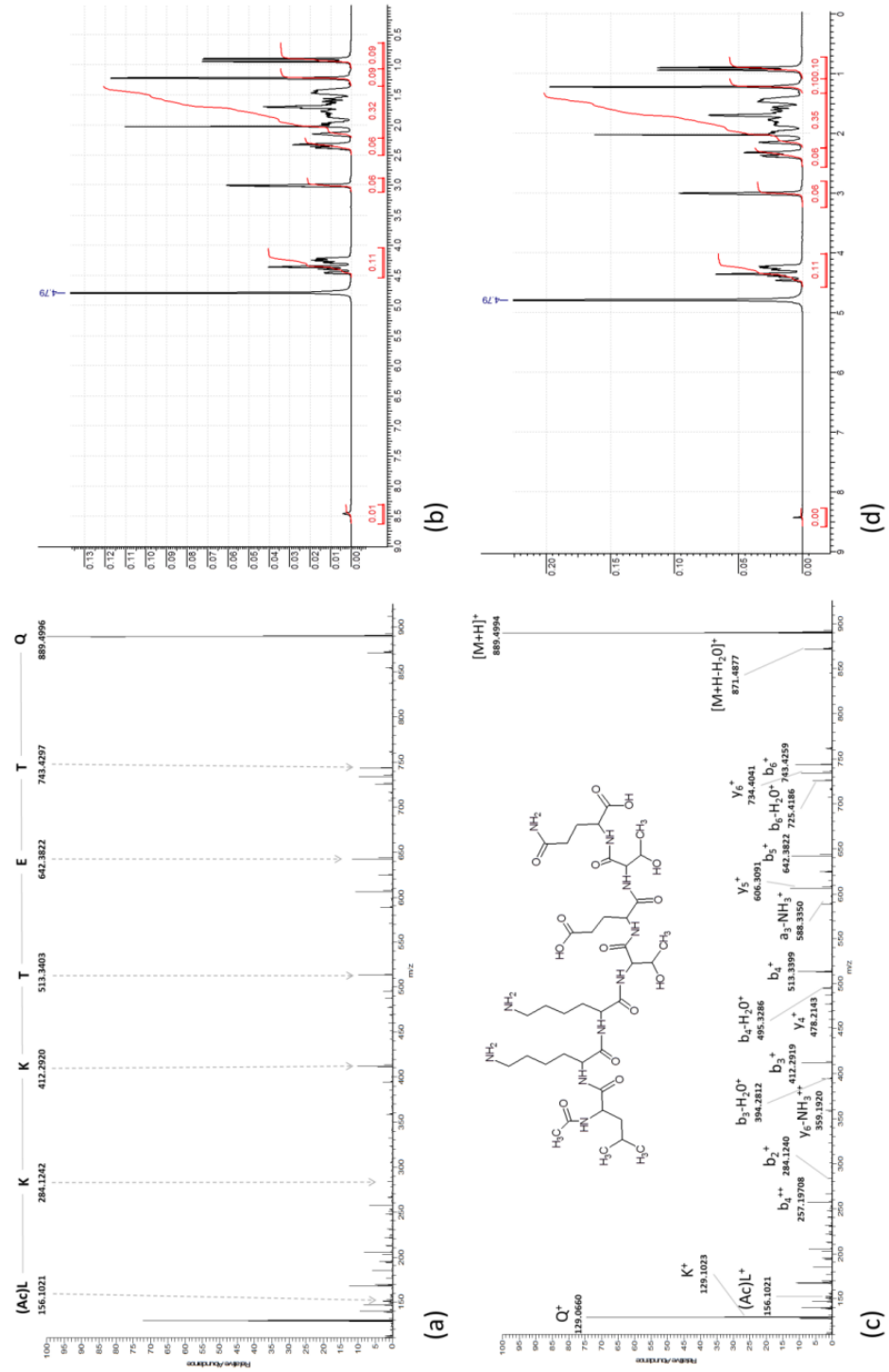

Figure 8.2 HCD MS/MS recorded at 20 eV (a) and 700 MHz-NMR (b) spectra of TB-500. The structure of the peptide was confirmed after synthesis of the Ac-Tb4(17-23), which presents identical MS/MS (c) and NMR (d) spectra.

Table 8.1 Identification of Ac-Tb4(17-23) based on matching between the experimental and *in silico* data on *b* and *y* ions from HCD experiments.

$(b)^+_{theor}$	$(b)^+_{exp}$	Δppm		Amino acid		$(y)^+_{theor}$	$(y)^+_{exp}$	Δppm
156.1019	156.1020	-0.6	1	(Acetyl)L	7	-	-	-
284.1969	284.1969	0.0	2	K	6	734.4043	734.4042	0.1
412.2918	412.2918	0.0	3	K	5	606.3093	606.3094	-0.2
513.3395	513.3396	-0.2	4	T	4	478.2144	478.2145	-0.2
642.3821	642.3817	0.6	5	E	3	377.1667	377.1670	-0.8
743.4298	743.4297	0.1	6	T	2	248.1241	248.1240	0.4
-	-	-	7	Q	1	147.0764	147.0765	-0.7

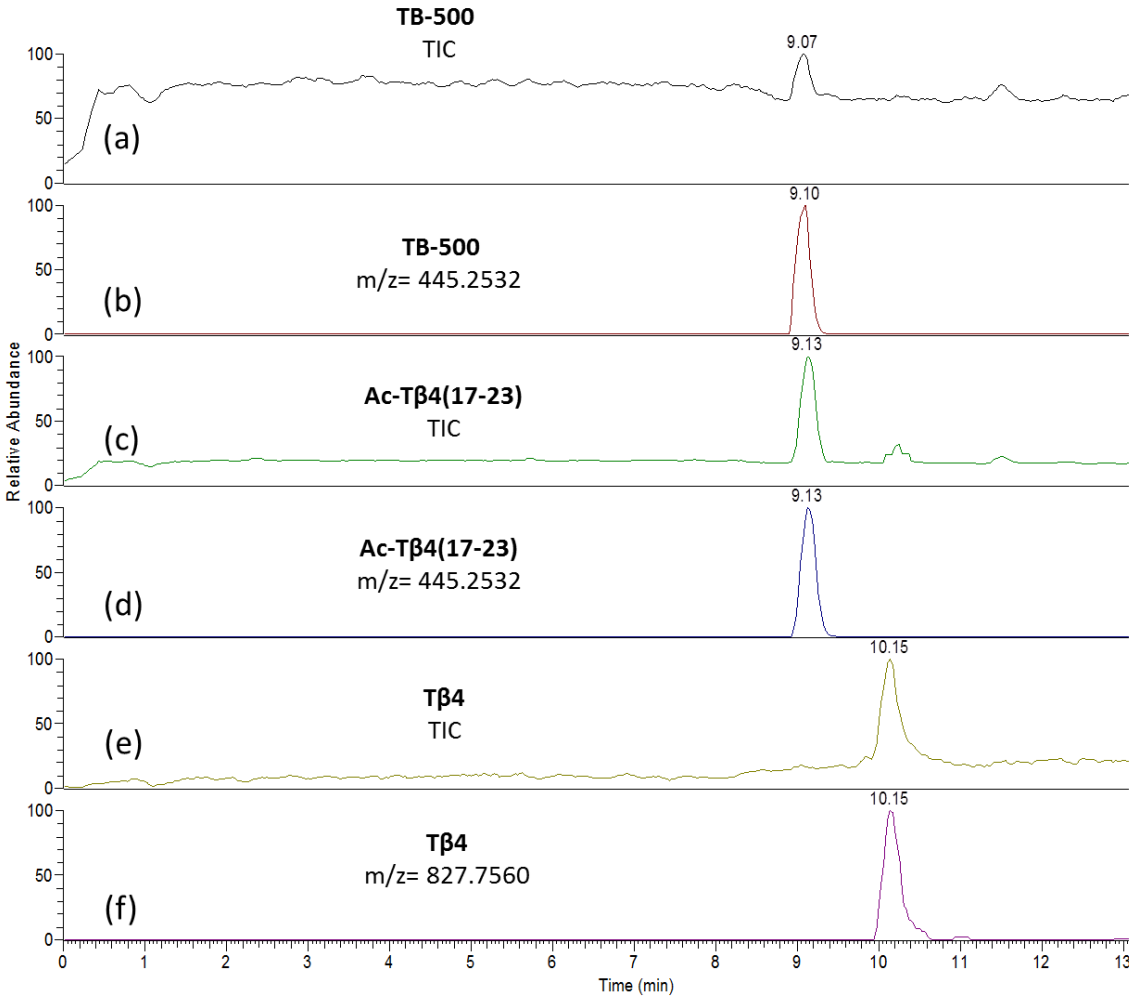

Figure 8.3 TIC and base peak chromatogram for TB-500 (a, b), the synthesized Ac-Tb4(17-23) (c, d), and Tb4 (e, f).

4.2 Synthesis of reference standards

The absence of reference standards for most of the black market peptides, including Ac-Tβ4(17-23), presents a problem in doping control concerning the fulfillment of compound identification criteria[12]. A major issue is the fact that mass spectrometry-based identification does not provide any indication on the stereochemistry of the amino acids in the unknown peptide. D-amino acids are commonly used to improve the pharmacokinetic profile of peptidic drugs by providing resistance to protease mediated degradation (e.g.: desmopressin) and thus it is not unlikely to find them in designer peptides.

From the analytical point of view, different stereochemistry of a molecule can theoretically reflect in different chromatographic behavior and MS fragmentation.

Therefore, synthesis of reference material is necessary both to provide essential identity confirmation of a new substance and as a quality control checks.

Luckily, synthesis of small peptides is a relatively straightforward process. The most common method is represented by solid-phase synthesis (SPPS). In this technique, the peptide chain is assembled in a stepwise manner while it is attached at one end to a solid support.

TB-500 was synthesized from the carbonyl group side (C-terminus) to amino group side (N-terminus). The C-terminal glutamine was covalently bound to polystyrene beads (Wang resin). Once the first amino acid was activated by removing its Fmoc protecting group, the peptide is obtained by repeated cycles of coupling-deblocking (Figure 8.4). After all the amino acids were coupled, the peptide underwent acetylation. Finally, after completion of the synthesis the first amino acid is cleaved from the solid support.

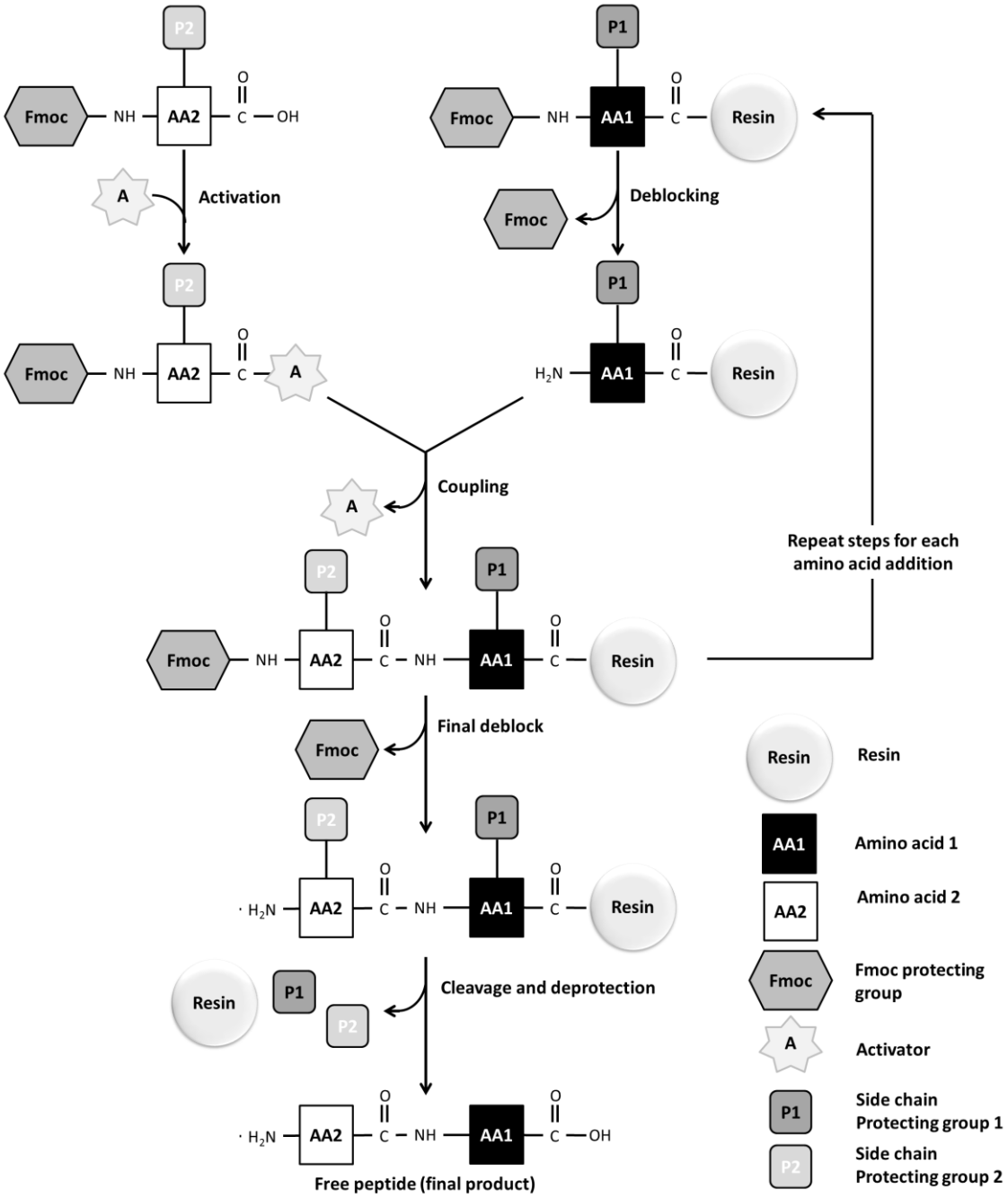

Figure 8.4. Schematization of basic SPPS steps.

After synthesis and before NMR analysis, the synthesized TB-500 was purified by preparative liquid-chromatography. Also the confiscated product underwent the same process in order to eliminate other components (excipients and possible impurities). As already shown in Figure 8.2b and 8.2d, NMR spectra confirmed TB-500 structure and stereochemistry.

4.3 *In vitro* metabolism studies and synthesis of TB-500 metabolites

The work from Ho *et al*.[13] showed that several TB-500 metabolites could be detected both in horse urine and plasma. Since TB-500 is a non-approved drug, administration studies on humans could not be carried out. Therefore, an investigation on TB-500 metabolism was performed using *in vitro* (human liver microsomes, S9 fraction, and fresh plasma) in order to evaluate the most probable metabolites. These models are extensively discussed in Chapter 9. Similarly to Ho *et al*., a sequential cleavage from the C-terminus was recorded, whereas the N-terminus is well protected by the acetyl group (Figure 8.7).

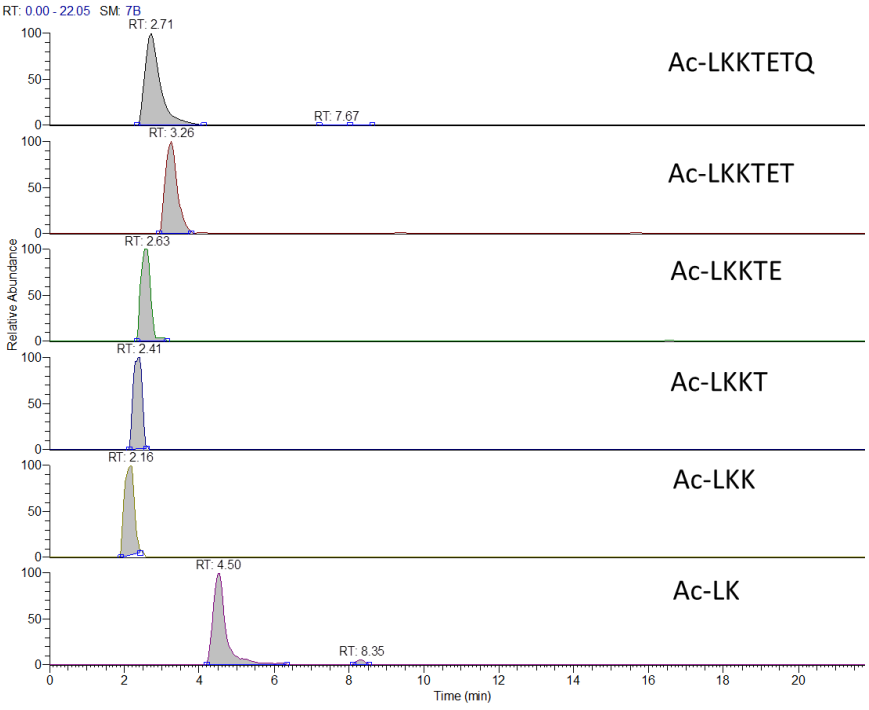

Figure 8.5 Results from incubation of TB-500 in fresh serum.

Finally, three metabolites were selected for synthesis and then implementation in routine analysis: Ac-LK, Ac-LKK and Ac-LKKTE. MS/MS spectra of these species are shown in Figure 8.6. These metabolites are those which are detectable for the longest time in horse urine, and at the same time very abundant both in the *in vitro* models (roughly estimated by comparing absolute area of the peaks). The implementation of these metabolites in the routine methods (Chapter 10) will increase the chances to detect misuse of TB-500.

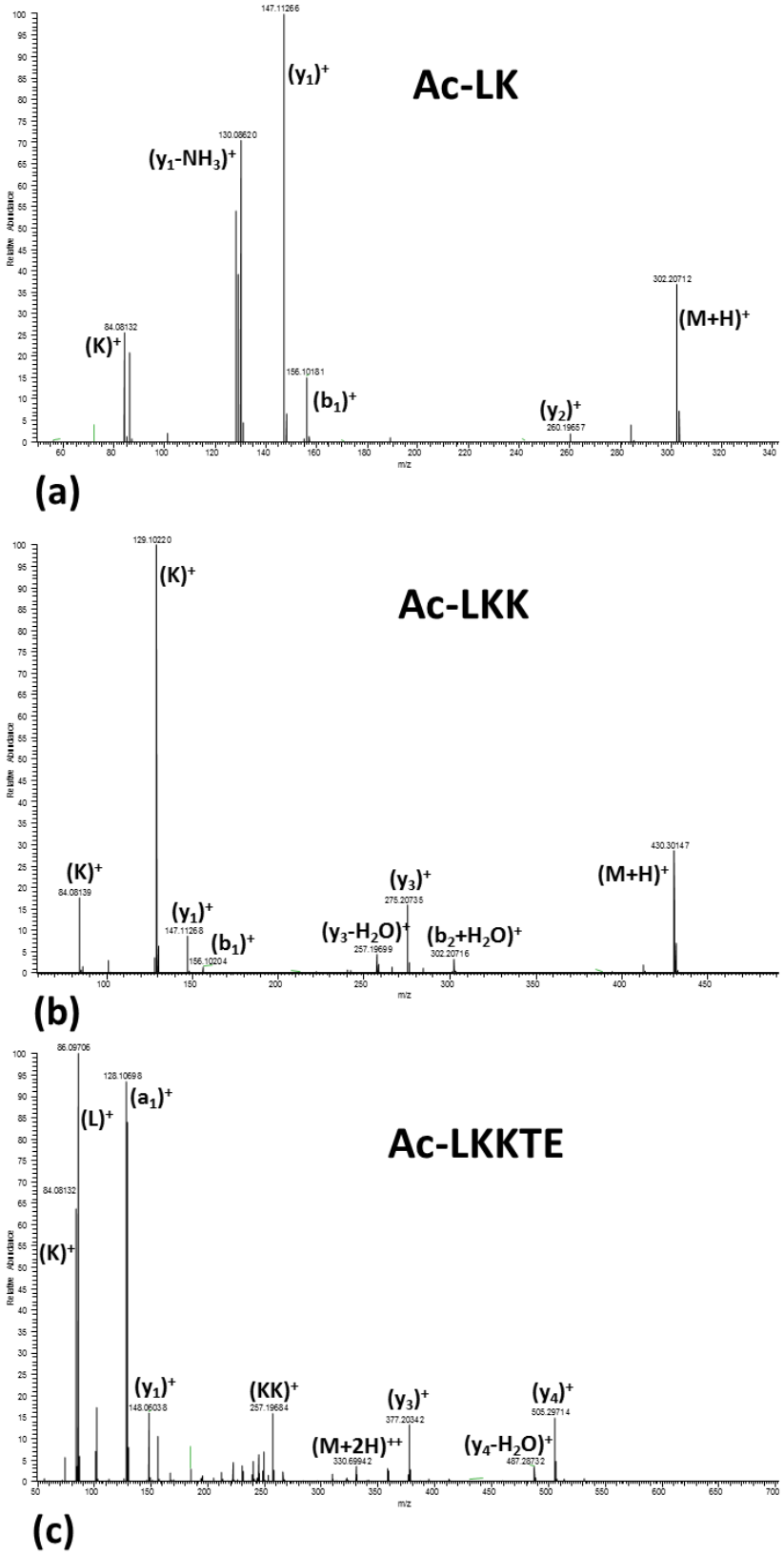

Figure 8.6 MS/MS spectra of the three synthesized TB-500 metabolites.

4.4 TB-500 and doping

TB-500 is one of several formulations available on the online market that contain a peptidic drug which is claimed to enhance sport performances. Although it is marketed only for veterinary use it is sold to non-veterinarians as well via the internet and there is anecdotal evidence that is probably used by athletes.

Ac-Tβ4(17-23) corresponds to the acetyl derivative of the major actin-binding site of Tβ4, that has been shown to promote angiogenesis, wound healing and hair growth *in vitro* and in animal models[14]. Therefore, the plausible rationale for use of TB-500 in doping lies in the role of Ac-Tβ4(17-23) in tissue growth and regeneration, which can be particularly important for recovery after intense workout or competition. The non-acetylated form of the peptide has been detected *in vivo* as an endogenous degradation product of Tβ4[14], whereas the acetylated form is not described. N-terminal acetylation is a common strategy in drug development of peptides to increase the half-life, and therefore the activity, of peptides[15]. This acetylation should allow for a clear differentiation between endogenous and exogenous Tβ4 in biological samples.

Though there are no pharmacokinetics data on TB-500, urinary excretion can be expected as well as urinary concentration in the femtomole range, similarly to other peptides.

Analysis of this peptide and its metabolites can be implemented in already existing methods for detection of small peptide hormones[16] (See Chapter 10).

5 Conclusions

Ac-Tβ4(17-23) was identified as the active content of the formulation TB-500. The identity was confirmed by synthesis of a reference standard. Moreover, three metabolites were also synthesized after *in vitro* metabolism studies.

TB-500 must be considered a product with doping potential and its misuse should therefore be monitored by anti-doping laboratories.

6 Acknowledgements

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Part IV - Peptide metabolism

Chapter 9

In vitro models for metabolic studies of small peptide hormones in sport drug testing.

Adapted from:

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1 Abstract

Peptide hormones represent an emerging class of potential doping agents. Detection of their misuse is difficult due to their short half-life in plasma and rapid elimination. Therefore, investigating their metabolism can improve detectability. Unfortunately, pharmacokinetic studies with human volunteers are often not allowed because of ethical constraints and therefore alternative models are needed. This study was performed in order to evaluate *in vitro* models (human liver microsomes and S9 fraction) for the prediction of the metabolism of peptidic doping agents and to compare them to the established models. The peptides which were investigated include desmopressin, TB-500, GHRP-2, GHRP-6, hexarelin, LHRH and leuprolide. Several metabolites were detected for each peptide after incubation with human liver microsomes, S9 fraction, and serum, which all showed endo- and exopeptidase activity. *In vitro* models from different organs (liver versus kidney) were compared but no significant differences were recorded. Deamidation was not observed in any of the models and was therefore evaluated by incubation with α-chymotrypsin.

In conclusion, *in vitro* models are useful tools for forensic and clinical analysts to detect peptidic metabolic markers in biological fluids.

2 Introduction

In recent years, the number of approved peptide therapeutics has increased significantly[1]. Contemporarily, peptides have become increasingly popular among elite and amateur athletes and bodybuilders to enhance their sport performances or to improve physical appearance[2]. Several small peptides (molecular weight < 2 kDa) with different pharmacological properties have reached the focus of being misused[2,3].

Since peptide drugs only reach low plasma and urine concentrations (< 1 ng/mL) due to their short half-life (generally < 12-24 hours) and low therapeutic doses, efficient testing of peptide hormone use is a difficult task[4–7].

Characterization of the metabolism of peptides can help to improve their detectability; in particularly characterization of long-term metabolites can increase the detection window. Unfortunately several of these peptides (e.g.: TB-500) are not approved drugs and therefore excretion studies involving humans are limited by ethical constrains. Therefore, most of the research has focused on the detection of the parent drug[6,7]. However, metabolites of the growth hormone releasing peptide 2 (GRHP-2) (AA 1-3, AA- 1-3(Amide), and AA 1-6) have been detected in human urine, allowing to increase the detection window for this peptide. AA 1-3 was first detected by Okano *et al*.[8] and confirmed by Thomas *et al*.[9], whereas the other two metabolites were described by Thomas *et al*. in a comprehensive study on the metabolism of GHRPs using *in vivo* (administration in rats) and *in vitro* (human serum and recombinant amidase) models[10]. More recently, detection of the metabolite AA 5-9 of the luteinizing hormone-releasing hormone (LHRH) analogue leuprolide has also been described[11].

The use of human liver microsomes (HLM) and/or S9 fraction for the metabolism of peptides has not been described so far, since these two models are generally used for small, non-peptidic molecules which show prevalent hepatic metabolism[12].

In this work, we have evaluated and compared the use of *in vitro* models (HLM, liver/kidney S9 fraction fresh serum, and the deamidating enzyme α-chymotrypsin) for the prediction of metabolism of prohibited small peptides. Seven peptides among those prohibited by WADA

were chosen for this study (Table 9.1). All analyses were performed by liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS).

Table 9.1 List of the peptides investigated in the study

Peptide	Amino acid sequence	Abbreviation	Pharmacologic/doping effect
Desmopressin	MrpYFQNCPdRG-NH_2	Mpa. Mercaptopropionic acid	Haemodilution, with alteration of blood passport parameters
TB-500	Ac-LKKTETQ		Tissue regeneration, angiogenesis
GHRP-2	dA(d-β-Nal)AWdFK-NH_2	Nal: naphylalanine	Increased growth hormone synthesis and secretion
GHRP-6	HdWAWdFK-NH_2		
Hexarelin	H(dMethW)AWdFK-NH_2	MethW: 2-methyltryptophan	
LHRH	PyrHWSYGLRPG-NH_2	Pyr: pyroglutami acid Et: ethyl group	Increased testosterone synthesis and secretion
Leuprolide	PyrHWSYdLLRPG-NH(Et)		

3 Materials and methods

3.1 Chemical and reagents

Desmopressin, LHRH, leuprolide and GHRP-2 were purchased from Bachem Ltd. (Bubendorf, Switzerland). GHRP-2, GHRP-6 and hexarelin were a kind gift from the Australian National Measurement Institute, and were synthesized by Auspep (Tullamarine, Australia). TB-500 was synthesized in-house (see Chapter 8)[13].

Peptide stock solutions (100 µg/mL) were prepared by dissolving the reference material in 2% HOAc and stored at −15 °C.

HPLC-grade water (H_2O), acetonitrile (ACN) and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Formic acid (FA) was purchased from Fisher Scientific

(Loughborough, UK). Acetic acid (HOAc) and sodium acetate p.a. were of analytical grade and were purchased from Merck (Darmstadt, Germany).

For *in vitro* incubations, phosphate buffer (pH 7.4), pooled human liver microsomes and liver S9 fraction, were from BD Bioscience (Erembodegem, Belgium). Kidney S9 fraction was purchased from (Lenexa, KS, USA). The gas used for mass spectrometry was nitrogen (Alphagaz2-grade) from Air Liquide (Desteldonk, Belgium).

Plasma and serum samples were obtained from the Belgian Red-Cross and left over doping control samples from blood analysis.

α-chymotrypsin (α-CT) from bovine pancreas was purchased from Sigma-Aldrich (Bornem, Belgium).

3.2 *In vitro* model with microsomes and S9 fraction

For microsomal incubation, 50 µL of each peptide stock solution were first evaporated to dryness at 40 °C. Dried standards were then reconstituted with 100 µL of 0.5 M phosphate buffer.

The reaction was initiated by adding 2.6 µL of HLM or S9 fraction (both from human liver and kidney), to obtain a total enzyme protein concentration of 0.5 mg/mL. Cofactors (NADPH generating system) were not added, unlike in typical experiments with non-peptidic molecules, since they are essential for the CYP system, but not for proteolytic enzymes.

The mixture was vortexed briefly and incubated at 37 °C for 6 h. To stop the incubation, 100 µL of ice-cold methanol was added and the tubes were transferred to an ice-bath for 15 min, and the sample was then centrifuged for 5 min at 12,000 g at 4 °C. Finally, the supernatant was transferred to a low-bind Eppendorf vial, ready for LC-MS analysis.

3.3 *In vitro* model with human serum

50 µL of peptide stock solutions were evaporated, redissolved in 200 µL of fresh serum and incubated at 37 °C for 2, 4, 6 and 24 h. Solid-phase extraction (SPE) was used for purification and concentration of the compounds of interest; Oasis® HLB 3cc (60 mg) extraction cartridges from Waters (Milford, MA, USA) were used. The cartridge was first activated with 2 mL of methanol and subsequently rinsed with 2 mL of water. Before loading, the sample was first diluted to 2 mL with water. After loading, the column was washed twice with 2 mL

of water. Then 1.2 mL of acetonitrile: water: formic acid (80: 15: 5) was used to elute the compounds from the column in a low-bind Eppendorf vial. After evaporation under a stream of oxygen-free nitrogen, the residue was redissolved in 100 µL of water/acetonitrile: formic acid (99:1:0.2 v/v/v) for LC–MS analysis.

3.4 *In vitro* incubation with α-CT

10 µl of peptide stock solutions were evaporated to dryness (40 °C) and then reconstituted in 100 µL α-CT solution (500 µg/mL in 25mM ammonium bicarbonate buffer, pH 8) in order to evaluate deamidation. Then, the sample was incubated under gentle stirring for 3 hours at 37 °C. Incubation was stopped with 2% acetic acid (100 µL) and samples were centrifuged and injected in the LC-MS system.

3.5 LC-MS instrumentation

Experiments were performed using a MS Pump Plus coupled with a Surveyor Plus autosampler (Thermo Scientific, Bremen, Germany). Separation was performed using a Zorbax 300SB-C18 reverse-phase column (1.0 mm×50 mm, 3.5 µm) from Agilent Technologies (Santa Clara, USA). The volume for injection was 10 µL. Mobile phase A was 0.2 % formic acid in water, and mobile phase B was 0.2 % formic acid in acetonitrile. The gradient elution program was as follows: 99 % A for 5 min, then linearly changed to 0 % A over 10 min and held for 3 min. This was followed by a return to the initial conditions in 0.1 min and equilibration for 6.9 min (total run time 25 min). The flow rate was constant at 50 µL/min.

MS detection was performed on a Q-Exactive mass spectrometer (hybrid triple quadrupole-Orbitrap mass spectrometer) from Thermo Scientific operating in positive mode. Full scan MS experiments were conducted for the first detection of metabolites, followed by MS/MS experiments of the most abundant molecular ions to confirm their structures. Conditions used were: sheath gas, 60 (arbitrary units); auxiliary gas, 30 (arbitrary units); capillary temperature, 350 °C; capillary voltage, 30 V; spray voltage, 4 kV; full-scan range, m/z 50–1000; high-collision dissociation energy, 30 eV; resolving power, 140 000; data acquisition rate, 1 Hz.

MS/MS experiments were performed at different normalized collision energy (NCE) values for different metabolites in order to maximize information from the spectra.

The online software Protein Prospector[14] was used for in silico prediction both of peptide metabolism and their fragmentation paths. In silico data were then compared with experimental results obtained by full scan MS and targeted MS/MS experiments.

4 Results and discussion

4.1 Metabolite characterization

Peptides were first incubated with the different media, then samples were processed as described above and analyzed by LC-HRMS. Moreover, negative control samples containing only HLM or only S9 fraction were also prepared and analyzed.

Metabolites were first identified in full scan MS experiments by extracting their exact *m/z* values of the pseudomolecular ions among those predicted *in silico*. Then, the identity of the metabolites was confirmed by targeted MS/MS experiments, as shown in Figure 9.1 for desmopressin (upper spectrum) and its metabolite AA 1-7 (lower spectrum).

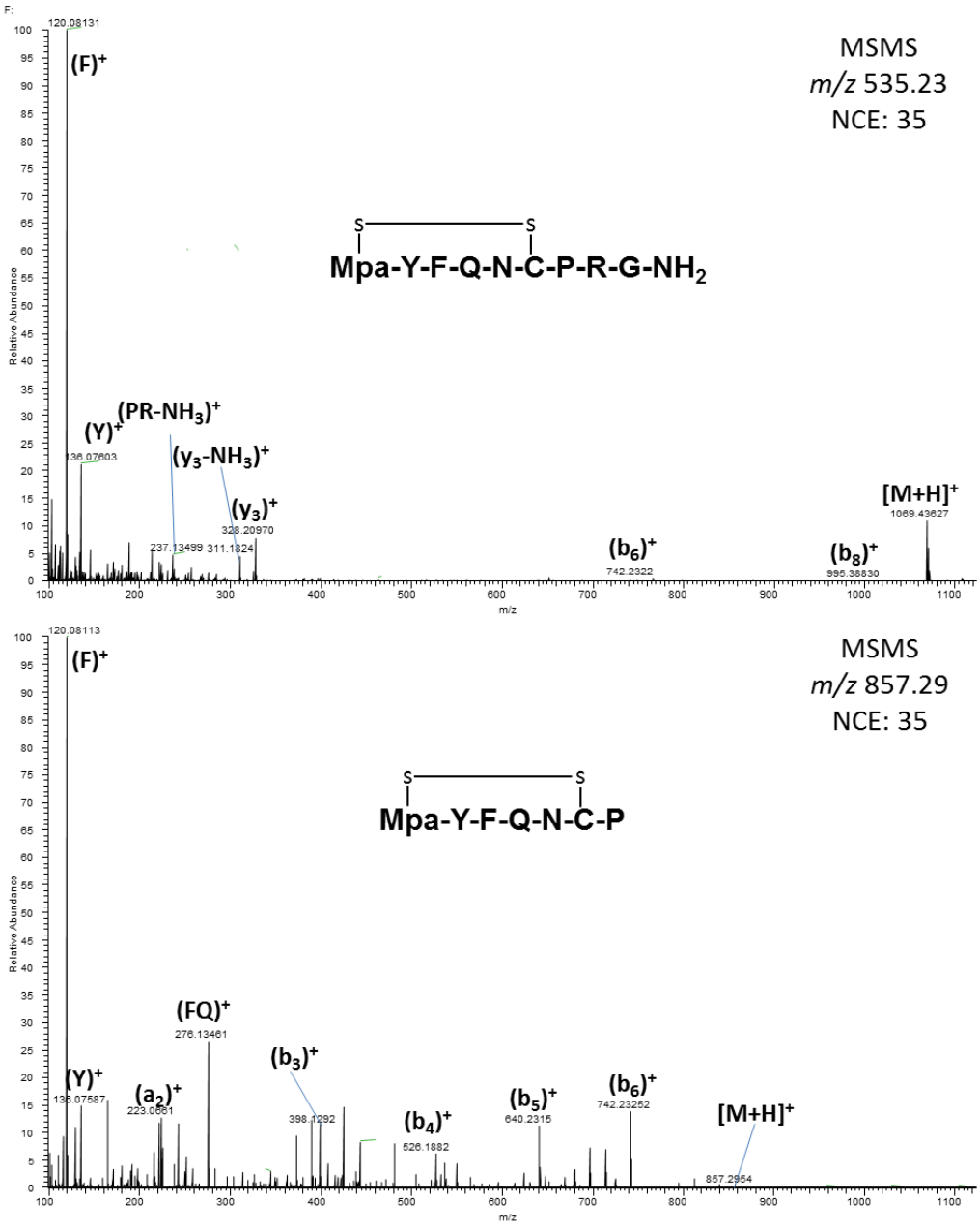

Figure 9.1 MS/MS spectra of desmopressin (upper spectrum) and its metabolite AA 1-7 (bottom) with a normalized collision energy (NCE)= 35..

Unspecific proteolysis via exopeptidases and endopeptidases was, as expected, the major metabolic pathway for peptides. Indeed, most common enzymatic degradation pathways for peptides involve cleavage of terminal amino acids (e.g. amino- and carboxypeptidases), which can break down peptides into monomers, and cleavage of non-terminal amino acids by endopeptidases (e.g. trypsin, chymotrypsin)[15].

Preliminary experiments showed that production of metabolites from HLM and S9 fraction reached a plateau after 6 hours, whereas the best results in serum (in terms of number and peak area of the detected metabolites) were obtained when samples were incubated for 24 hours (data not shown).

Whereas the use of serum as an *in vitro* model has already been described[10] and the activity and abundance of serum proteases is well known, hepatic metabolism of peptides has not yet been intensively investigated. One of the reason may be the fact that peptidic drugs generally undergo very intense first-pass metabolism, both in the gastro-intestinal tract but also in the liver. Therefore, oral route is generally avoided[16].

However, although the liver does not usually have a primary role in peptide metabolism, several classes of liver proteases have been described[16,17]. For example, mitochondrial proteolytic system has an important role in quality control of mitochondrial proteins, but it is legitimate to hypothesise that it can also catalyse (at least *in vitro*) degradation of peptidic drugs[17]. A few work have addressed hepatic metabolism of peptides: *in vivo* hepatic metabolism of insulin had been investigated[18], and homogenized horse liver was used for investigation of TB-500 metabolism in horse[19]. A complete list of the metabolites detected for all the investigated compounds in the different models is summarized in Table 9.2. As an example, chromatograms from GHRP-2 incubation experiments are shown in Figure 9.2. For completeness, results from preliminary experiments concerning incubation in plasma (blood was collected in EDTA tubes) are also shown. In general, results from incubation in plasma demonstrated very limited metabolization in this model. It is known, indeed, that EDTA tubes can limit proteolysis as already mentioned by Thomas *et al*.[10].

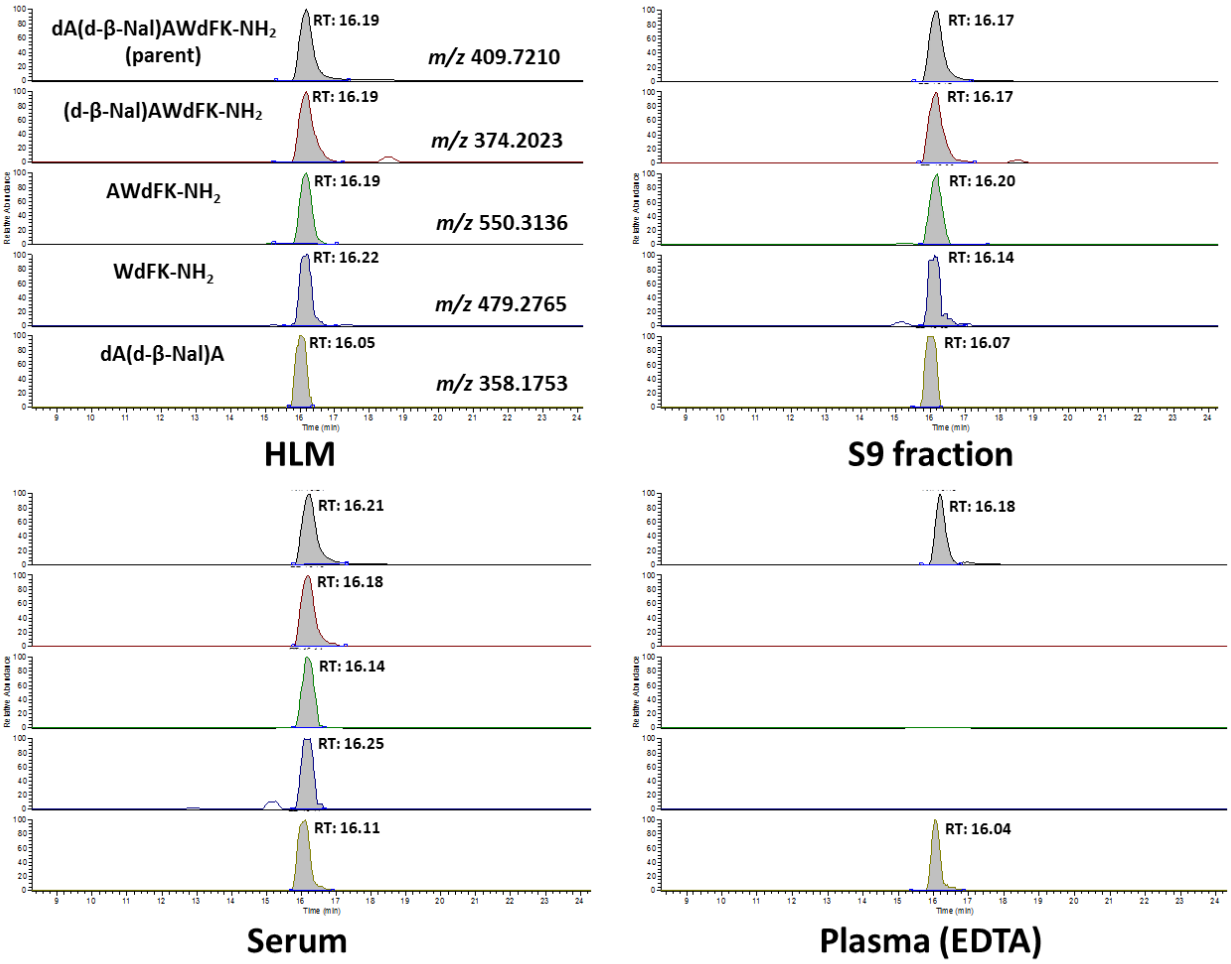

Figure 9.2 Extracted ion chromatograms for GHRP-2 and four GHRP-2 metabolites analyzed after incubation with HLM, S9 fraction, serum, and plasma.

Table 9.2 List of the metabolites identified for all the seven peptides with the different models. Mass accuracy was lower than 4 ppm in all cases. LHRH metabolism was investigated with both liver and kidney S9 fraction; the two models produced the same metabolites(*).

Peptide	Metabolite	Theoretical *m/z*	Charge	HLM	S9 fraction (liver)	Serum	In vivo [reference]
Desmopressin	MrpYFQNCPRG-NH2 (parent)	535,2187	++	D	D	D	human [6,7]
	MrpYFQNCPRG	535,7192	++	detected after incubation			ND
	MrpYQNCPR	507,2120	++	D	D	D	ND
	MrpYFQNCP	857,2916	++	D	D	D	ND
	MrpYQNCPRG-NH2 (loss of Fby double cleavage)	461,1857	++	D	D	D	ND
TB-500	Ac-LKKTETQ (parent)	445,2531	++	D	D	D	horse [18]
	Ac-LKKTET	381,2238	++	D	D	D	horse [18]
	Ac-LKKTE	330,7000	++	D	D	D	horse [18]
	Ac-LKKT	531,3501	+	D	D	D	horse [18]
	Ac-LKK	430,3024	+	D	D	D	horse [18]
	Ac-LK	302,2074	+	D	D	D	horse [18]
GHRP-2	dA(d-β-Nal)AWdFK-NH2 (parent)	409,7210	++	D	D	D	rat, human [8,9,10]
	dA(d-β-Nal)AWdFK	410,2198	++	detected after incubation			rat, human [10]
	dA(d-β-Nal)A	358,1753	+	D	D	D	rat, human [10]
	dA(d-β-Nal)A-NH2	357,1801	+	D	D	D	rat, human [10]
	(d-β-Nal)AWdFK-NH2	374,2023	++	D	D	D	ND
	AWdFK-NH2	550,3136	+	D	D	D	ND
	WdFK-NH2	479,2765	+	D	D	-	ND
GHRP-6	HdWAWdFK-NH2 (parent)	437,2296	++	D	D	D	rat [10]
	HdWAWdFK	437,7216	++	detected after incubation			rat [10]
	dWAWdFK-NH2	368,1924	++	D	D	D	rat [10]
	dWAWdF	609,2823	+	D	D	D	rat [10]
	AWdFK-NH2	550,3136	+	D	D	D	ND
	WdFK-NH2	479,2765	+	D	D	-	ND
Hexarelin	H(dMethW)AWdFK-NH2 (parent)	444,2374	++	D	D	D	rat [10]
	H(dMethW)AWdFK	444,7293	++	detected after incubation			rat [10]
	(dMethW)AWdFK-NH2	375,7079	++	D	D	D	rat [10]
	AWdFK-NH2	550,3136	+	D	D	D	ND
	WdFK-NH2	479,2765	+	D	D	D	ND
LHRH	PyrHWSYGLRPG-NH2 (parent)	591,7914	++	D	D*	D	human [19]
	PyrHWSYGLRPG	592,2910	++	detected after incubation			ND
	HWSYGLRPG-NH2	536,2778	++	D	D*	D	ND
	WSYGLRPG-NH2	467,7483	++	D	D*	D	ND
	SYGLRPG-NH2	374,7087	++	D	D*	D	ND
	YGLRPG-NH2	331,1926	++	D	D*	D	ND
	GLRPG-NH2	498,3147	+	D	D*	D	ND
	LRPG-NH2	441,2932	+	D	D*	-	ND
	PyrHWSYGLRP	563,7727	++	D	D*	-	ND
	PyrHWSYGLR	515,2463	++	D	D*	-	ND
	PyrHWSYGL	437,1958	++	D	D*	-	ND
	PyrHWSYG	380,6537	++	D	D*	-	ND
	HWSYGLRP	508,2591	++	D	D*	-	ND
Leuprolide	PyrHWSYdLLRP-NH(Et) (parent)	603,3307	++	D	D	D	human [11]
	PyrHWSYdLLRP	591,804	++	D	D	D	ND
	PyrHWSYdLLR	543,2776	++	D	D	D	ND
	PyrHWSY	352,143	++	D	D	D	ND
	PyrHW	453,1834	+	D	D	D	ND
	HWSYdLLRP-NH(Et)	549,8153	++	D	D	D	ND
	WSYdLLRP-NH(Et)	481,2858	++	D	D	D	ND
	SYdLLRP-NH(Et)	388,2461	++	D	D	D	ND
	YdLLRP-NH(Et)	344,7301	++	D	D	D	human [11]
	dLLRP-NH(Et)	525,3896	+	D	D	D	ND
	LRP-NH(Et)	412,3056	+	D	D	D	ND
	SYdLLR	326,1949	++	D	D	-	ND

Desmopressin is a synthetic analogue of the antidiuretic hormone (ADH). The deamination on the cysteine in position 1, the use of D-Arginine in position 8, and the amidation of the carboxyl terminus were made with the purpose of protecting from enzymatic degradation and, thus, increasing half-life. Nevertheless, two metabolites were generated by cleavage at the carboxyl side of arginine and proline residues. Deamidation at the C-terminus was observed after incubation with α-CT (see further in the text). One of the metabolites

detected presents loss of phenylalanine by double cleavage. The formation of this species, catalysed by α-chymotrypsin, has been described previously[20].

TB-500 (N-term acetylated 17-23 fragment of thymosin β4) showed serial cleavage at the C-terminus (see Chapter 8, Figure 8.5), whereas acetylation of the leucine seemed to provide efficient protection of the N-terminus. Results were similar to those described by Ho *et al*. in the horse using horse liver homogenate as *in vitro* model[19].

Metabolism of GHRPs has been first investigated by Okano *et al*.[8] and then extensively described by Thomas *et al*.[9,10]. They observed action of amidases, oligopeptidases and both carboxy- and aminoexopeptidases after *in vivo* (administration to rats) and *in vitro* (incubation with human serum and recombinant amidase) experiments. Then the incubation results were used and compared with the administration of GHRP-2 in humans. Results yielded the discovery of two additional human metabolites in urine (AA 1-3(Amide) and AA 1-6). After incubation of GHRP-2, GHRP-6 and hexarelin with human serum, the metabolites detected were similar to those observed by Thomas *et al*.[10], in a rat model and after administration to a human (only GHRP-2) [10]. It was also observed that the GHRPs metabolites were produced by HLM and S9 fraction. Moreover, additional metabolites were detected for GHRP-2 (3), GHRP-6 (2) and hexarelin (2) in all the three models in this study (Table 9.2).

The other growth hormone secretagogues GHRP-1, GHRP4, GHRP-5, alexamorelin and ipamorelin could not be included in this study since no reference standards were available.

All the substances mentioned so far were chemically modified analogues of endogenous peptides created with the aim of improving pharmacokinetic properties and, thus, therapeutic effect. All these peptides contain at least a modified amino acid that can provide unambiguous identification in the frame of sport drug testing.

Other prohibited peptide drugs, such as LHRH, do not have modified AA, but their sequence corresponds to the endogenous hormones or to a portion of it. In this case, it is important to know whether they are present physiologically at detectable concentration in plasma or urine. LHRH is only secreted locally and it is not found in urine, as confirmed by the work of Thomas *et al*.[21].

In this study, it was possible to verify how LHRH is indeed extensively metabolised by peptidases, showing serial cleavage both at the C- and N-terminus and also C-term deamidation. *In vitro* models showed a significantly higher number of LHRH metabolites compared to serum (Table 9.2). Since LRHR has very short half-life, several analogues (e.g. leuprolide) have been in fact designed with AA substitutions at position 6 and/or 10[22]. Leuprolide has both glycine residues in position 6 and 10 substituted respectively with a D-leucine and an ethylamino moiety linked to the proline in position 9. A total of ten metabolites were identified in HLM and S9 fraction, whereas only nine were found in serum, as the metabolite AA 4-8 was not detected in this latter model. The metabolite AA 5-9 described *in vivo*[23] was detected in all three models.

4.2 Hepatic vs. renal *in vitro* models

Kidneys are another main site for peptide metabolism. In particular, LHRH has been described as being extensively metabolised in kidney[23], and therefore it was used as model peptide for this investigation. LHRH was incubated with both liver and kidney S9 fraction for 6h, with the same S9 protein concentration (final concentration of 0.5 mg/mL) and metabolites were then identified by LC-MS.

No differences were noticed in the number of metabolites (summarized in Table 9.2). It is interesting to notice that for both models the sequential cleavages from C- and N-term stopped at the same site (the carboxyl side of the Gly_5 residue).

Thus, both for the liver and kidney models identical results for LHRH metabolism were obtained in our study. However, as a general rule, it is suggested to evaluate S9 fraction from different organs especially when a particular metabolism site is likely to have a main role.

4.3 Deamidation

Among the peptides investigated in this study, desmopressin, LHRH, GHRP-2, GHRP-6, and hexarelin have an amidated C-terminus. Deamidation results in a positive mass shift of +0.9852 Da. The mass shift of the deamidated metabolites is almost identical to that of the peak of the second isotope of the amidated species (mass shift +1.0046). Nevertheless, the two peaks have Δmass= 0.0194 corresponding to a Δppm= 38.8 (measured at 500 Da), and

can therefore be separated using a high resolution mass spectrometer with a resolving power > 25000, as shown in Figure 9.3. After incubation with HLM, S9 fraction and serum, the deamidated species was not detected for any of the peptides. Therefore this reaction was studied by using the enzyme α-CT, a protease with amidase activity. Deamidation was observed for all the five peptides after incubation with α-CT (Figure 9.4).

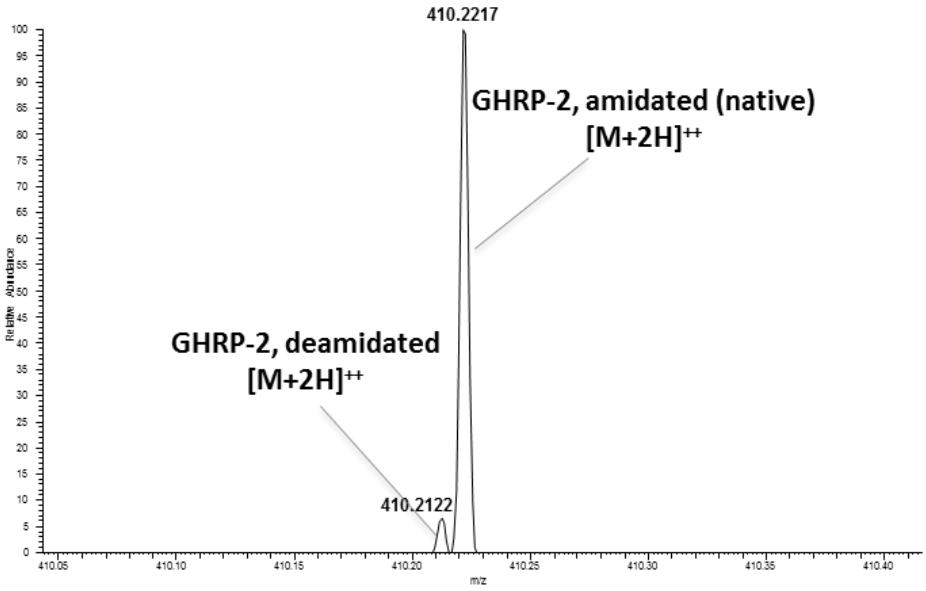

Figure 9.3 Amidated (second isotope) and deamidated (first isotope) GHRP-2 MS peaks at resolution= 140 000.

Thus, incubation with deamidating enzymes is preferred to HLM, S9 fraction and serum to evaluate deamidation. Deamidation of GHRPs was also described by Thomas *et al*.[10] by incubation with amidase. In that case, the GHRP-2 deamidated metabolite was also detected in human urine suggesting that C-term deamidation is a major degradation pathway for this peptide. This approach is less complex than the one used by Thomas *et al*.[10] to discriminate between the native and the deamidated peptide, since the method of Thomas et al.includes LC fractionation and evaluation of positive and negative response.

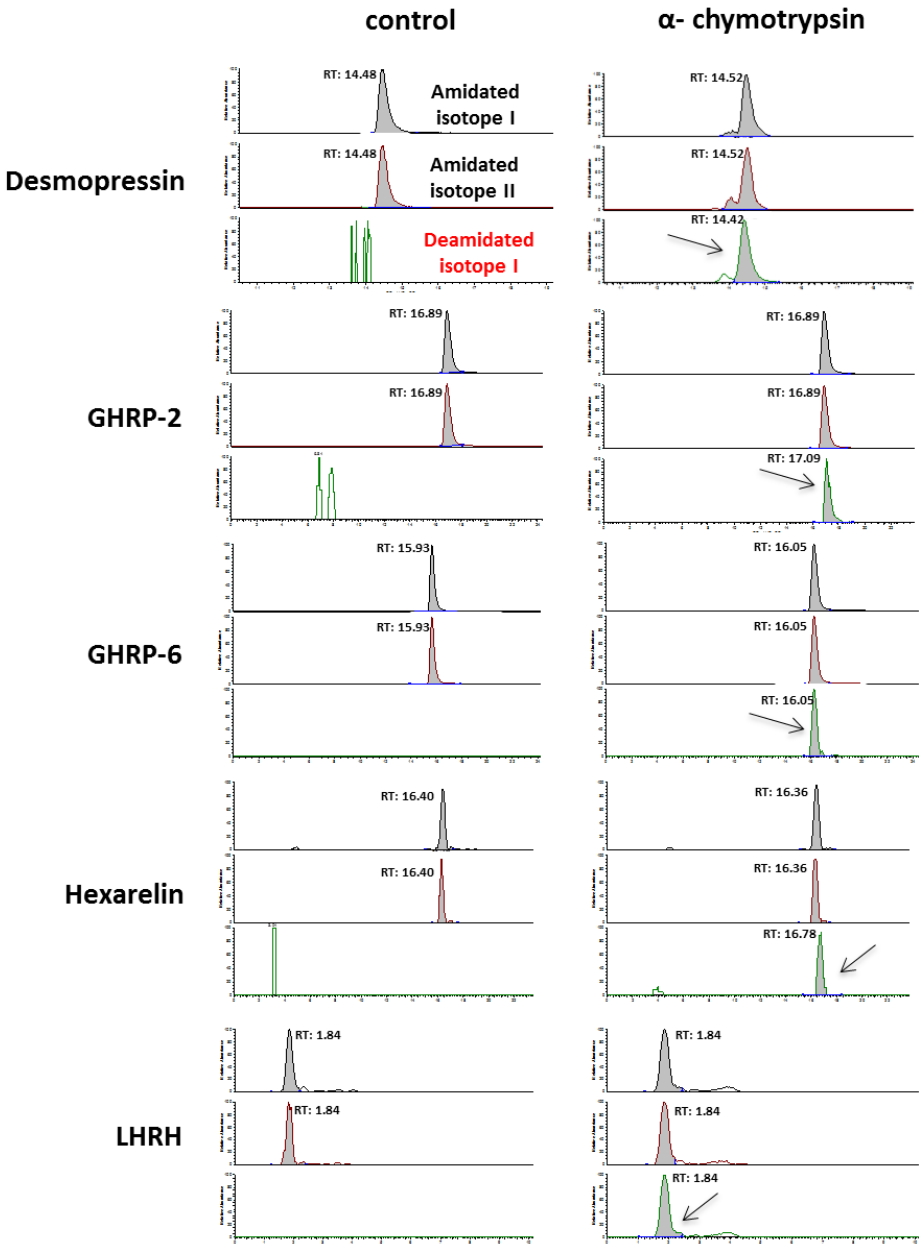

Figure 9.4 After incubation with α-CT, deamidation at the C-terminus was observed, as shown by extracted ion chromatograms for first (upper chromatogram) and second (middle row) isotopes of the molecular ions of the native peptide, and first isotope of the deamidated peptides (bottom). The latter was not detected in absence of the enzyme (control samples).

4.4 Cost effectiveness of the different models

Provided the access to fresh whole blood, serum is less expensive than S9 fraction and HLM. Moreover, incubation with serum is straightforward and doesn't require use of other

(expensive) reagents. On the other hand, serum requires additional costs of the SPE-based sample preparation to purify the samples, whereas *in vitro* experiments require only centrifugation of the incubation solution to precipitate HLM/S9 corpuscolate.

Purification of serum yields additional issues such as extraction recovery and interferences from a peptide-rich matrix. These interferences (e.g.: endogenous di-, tripeptides from serum which are identical to a portion of the sequence of the drug) could also generate, theoretically, false positive results in the detection of metabolites, which are less likely to occur with HLM and S9 fraction.

5 Conclusions

HLM and S9 fraction generated metabolites that were described *in vitro* (fresh serum) and *in vivo* (administration to human and rat model) in previous studies. Hence, HLM and S9 fraction are not only a valid tool to study *in vitro* metabolism of small, non-peptidic drug, but also for small peptide hormones. However, none of the models is suitable for evaluation of deamidation: to this purpose, deamidating enzymes (e.g.: α-CT) must be used. Liver and kidney models produced the same metabolites: however, it is recommended to consider both for metabolism studies, particularly when information on main site of metabolization for a particular class of compound.

In vitro models can potentially be used not only by the forensic analyst for the identification of better markers of doping with peptide hormones, but also by the clinical analysts as preliminary screening tools for the discovery of potential markers for peptides in biological fluids, especially plasma and urine. Moreover, they could be used in preclinical research during the screening process, in order to monitor metabolic stability of drug candidates, similarly to what is already being done for small molecules with “classic” *in vitro* models.

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Part V - Detection of small peptide hormones

Chapter 10

Development of a comprehensive UHPLC-HRMS screening method for the analysis of small peptide hormones and their metabolites in human urine

Adapted from:

S Esposito, K Deventer, L Geldof, P Van Eenoo. Doping control analysis of small peptides: an update. Recent Advances in doping analysis (22). Proceedings of the Manfred Donike Workshop. 32nd Cologne Workshop on Dope Analysis, 2014, Accepted/In press.

1 Abstract

The final goal of the thesis was to implement analysis of small peptides in routine. To this purpose, a UHPLC-HRMS screening method was developed for the analysis of 31 small peptides, including desmopressin and two other vasopressin analogues, TB-500, 8 GHRPs, LHRH and 6 LHRH analogues, and AOD9604. The method includes also 3 TB-500 metabolites, 5 GHRPs metabolites, and 3 LHRHs metabolites.

Sample preparation consisted of solid-phase extraction with weak-cation exchange cartridges. Detection was achieved on a Orbitrap® Q-Exactive HRMS in targeted SIM mode monitoring peptide pseudomolecular ions. Contemporarily, full scan analysis was performed to allow retrospective analysis. The method was successfully validated, with limits of detection were in the range 20-500 pg/mL. The method uses only 1 mL of urine and allows for screening analysis of 25-30 samples per day.

2 Introduction

As described in this thesis, the number and the variety of small peptide hormones which are subject of misuse in sport have grown exponentially in the last years. The list of small peptides and related metabolites) can currently count over 30 molecular entities (Table 10.1).

LHRH was the first compound whose detection has been investigated[1] as the pulsatile administration of this peptide can increase testosterone levels. Numerous LHRH agonists have been developed to improve pharmacological profile and are prohibited as well[2].

GHRPs, which are growth hormone secretagogues acting as synthetic ghrelin mimetics, represent the most investigated class of small peptidic doping agents, with numerous works describing their identification, detection and metabolism[3–7].

Desmopressin is the most representative synthetic analogue of arginine vasopressin (also known as antidiuretic hormone). It is considered a masking agents since it can artificially alter hematological parameters monitored in the framework of the biological passport[8].

More recently, misuse of the thymosin beta 4 fragment TB-500[9] and the growth hormone fragment AOD9604 has been described[10].

Additionally, several metabolites have been described over the years for GHRPs[6], LHRH agonists[11], and TB-500 (see Chapter 9). Some additional metabolites were characterized *in vitro* during unpublished studies.

Doping control laboratories have put a big effort on the analysis of this class of compounds. As a result of their work, general protocols for analytical methods, metabolic studies and synthesis of reference standard material have been established[3-13].

Although these peptides have different structures and properties, they share similar pharmacokinetics, analytical issues (e.g.: adsorption to surfaces), and chromatographic and mass spectrometric behavior. In a first phase, single-analyte or single-class methods were

published[1,4,12,13]. As the number of small prohibited peptides increased, the attention was focused on the development of multiclass methods.

Table 10.1 List of small peptides and metabolites.

Class	Peptide	Elemental composition	Molecular mass
AVP analogues	Desmopressin	C46H64N14O12S2	1068,4269
	Lys-vasopressin	C46H65N13O12S2	1055,4317
	Terlipressin	C52H74N16O15S2	1226,4961
TB-500	TB-500	C38 H69N10O14	889,4995
	TB-500 M1 (1-2)	C14H27N3O4	301,2001
	TB-500 M2(1-3)	C20H40N5O5	430,3029
	TB-500 M3 (1-5)	C29H54N7O10	660,3932
GHRPs	GHRP-1	C51H62N12O7	954,4860
	GHRP-2	C45H55N9O6	817,4721
	GHRP-2 M1 (1-3)	C19H23N3O4	357,1682
	GHRP-4	C34H37N7O4	607,1292
	GHRP-5	C43H46N8O6	770,3546
	GHRP-6	C46H56N12O6	872,4445
	GHRP-6 M1 (2-5)	C34H36N6O5	608.2747
	GHRP-6 M2 (2-6)	C40H50N9O5	736,3934
	Alexamorelin	C50H63O7N13	957,4972
	Ipamorelin	C38H49N9O5	711,3851
	Hexarelin	C47H58N12O6	886,4607
	Hexarelin M1 (1-3)	C21H26N6O4	426,2003
	Hexarelin M2 (2-5)	C35H38N6O5	622,2903
LHRH agonists	LHRH	C55H74N16O14	1181,4315
	Leuprolide	C59H84N16O12	1208,6455
	Leuprolide M1 (5-9)	C34H54N9O5	668,4248
	Leuprolide M3 1(-3)	C22H25N6O5	452,4703
	Buserelin	C60H86N16O13	1238,6560
	Deslorelin	C64H83N17O12	1281,6407
	Goserelin	C59H84N18O14	1268,6415
	Nafarelin	C66H83N17O13	1321,6356
	Nafarelin M1 (5-10)	C66H83N17O13	800,4334
	Triptorelin	C64H82N18O13	1310,6308
GH fragments	AOD 9604	C78H123N23O23S2	1812,8588

In 2012, Thomas *et al*. published a method for the determination in human urine of 11 small peptides, including desmopressin, LHRH and 9 GHRPs[5]. The method was based on WCX SPE purification and nanoLC-HRMS detection. The method showed the potential for allowing simultaneous analysis of chemically and pharmacologically unrelated small peptides.

In this work, we have developed a comprehensive screening method for detection of small peptide hormones, expanding the array of substances to other peptides and classes, including other LHRH analogues, TB-500, AOD9604, and additional metabolites for a total number of 31 compounds, including 11 metabolites. Sample preparation was based on solid-phase extraction (SPE), while detection was performed on a UHPLC-HRMS system.

3 Materials and methods

3.1 Chemicals and reagents

All the chemicals and solvents used for sample pretreatment and chromatography were analytical grade or HPLC grade. Desmopressin was a kind gift from Ferring Pharmaceuticals (Malmö, Sweden). AVP and [deamino-Cys1, Val4, D-Arg8]-vasopressin were purchased from Sigma Aldrich (Saint Louis, USA). [Lys8]-vasopressin and terlipressin were purchased from Selleck Chemicals LLC (Houston, USA).

All GHRPs, LHRH analogues and their metabolites were a kind gift from the Australian Sports Drug Testing Laboratory (Sydney, Australia) and were synthesized by Auspep. AOD9604 was a kind gift from the Centre for Preventive Doping Research, German Sport University (Cologne). TB-500 and its metabolites were synthesized in-house (see Chapter 9). Acetonitrile (ACN), water (H_2O) and glacial acetic acid (HOAc) were purchased from JT Baker (Philipsburg, USA); methanol (MeOH) was purchased from Fisher Scientific (Aalst, Belgium); formic acid (FA) was purchased from Sigma-Aldrich. Sodium acetate was purchased by VWR International (Leuven, Belgium).

Stock (100 μg/mL) and working solution (50 ng/mL) were all prepared using 2% HOAc aqueous solutions.

Internal standard (ISTD) solution contained [deamino-Cys1, Val4, D-Arg8]-vasopressin, ^{13}C,^{15}N GHRP-2 metabolite (AA-1-3) and LHRH(2-10) at a concentration of 100 ng/mL in 2% HOAc.

Only low binding microcentrifugal vials, type Eppendorf (Eppendorf, Hamburg, Germany) and low retention pipette tips (Sorenson Biosciences, Salt Lake City, USA) were used.

3.2 Sample preparation

Sample preparation was based on previous works[5,14]. Urine (1 mL) was spiked with 30 µl of ISTD solution. Then, 1 mL of acetate buffer (1M, pH 5.2) was added and the samples were centrifuged (4100 g, 10', 20 °C). SPE purification was performed with Strata® X-CW (30 mg) cartridges, purchased from Phenomenex (Torrance, USA). The column was first activated with 1 mL of MeOH and then rinsed with 1 mL of H_2O. After the sample was loaded, the column was washed first with 1 mL of H_2O, then with 1 mL of 60: 40 H_2O:MeOH mixture and 1 mL MeOH. Finally, samples were eluted with 1.20 mL of ACN: H_2O: FA 80: 10: 10. The eluate was subsequently evaporated to dryness with a centrifugal evaporator (45°C, 240 g, 1-2 h), and then dissolved in 120 µl of ACN: H_2O: FA 99: 0.8: 0.2 prior to LC-MS analysis.

3.3 Liquid chromatography

The liquid chromatographic system consisted of an Accela LC (Thermo Scientific, Bremen, Germany) equipped with degasser, Accela 1250 UPLC pump, autosampler thermostated at 10 ºC and a heated column compartment at 35 ºC.

The LC separation was performed on a Agilent Zorbax RRHD C8 (50x2.1 mm, 1.8 µm) at a flow rate of 50 µL min-1. The aqueous solvent (A) consisted of 0.2% of FA in H_2O, and the organic phase (B) was acetonitrile with 0.2% FA. The gradient started at 1 % B for 5 min, rose to 35 % B in 20 min, rose to 90 %B in 2 minutes and was followed by requilibration at 1 % B for 8 min, with a resulting overall runtime of 35 min.

3.4 Mass spectrometry

The LC effluent was pumped to an Q-Exactive benchtop quadrupole Orbitrap tandem mass spectrometer (Thermo Scientific, Bremen, Germany) operated in the positive mode and

equipped with an electrospray ionization (ESI) source. Nitrogen sheath gas flow rate and auxiliary gas were set at 60 and 30 (arbitrary units), respectively. The capillary temperature was 250 °C, the spray voltage 4 kV or -4 kV and the capillary voltage 30 V in positive or negative ion modes. The instrument operated both in full scan (FSMS) mode from *m/z* 300 to 1200 and in targeted single ion monitoring (tSIM) at 70,000 resolving power. tSIM experiments were performed with an isolation window of 2.5 Da using an inclusion list with all target analytes.

The automatic gain control (AGC) was 10e6. The Orbitrap performance in both positive and negative ionization modes was evaluated daily and external calibration of the mass spectrometer was realized with Exactive Calibration Kit solutions (Sigma–Aldrich, St. Louis, USA and ABCR GmbH & Co. KG, Karlsruhe, Germany).

3.5 Method validation

In accordance with Eurachem validation guidelines[15], 10 different blank human urine samples, were spiked at different levels (20, 50, 100, 500 pg/mL) to determine the limit of detection (LOD). The LOD was defined as the lowest level at which a compound could be identified in all 10 urine samples with one diagnostic pseudomolecular ion present with a signal-to-noise (S/N) ratio greater than 3 and a retention time difference of less than 0.2.

Specificity was tested during the validation procedure. The 10 blank urine samples used for determining detection capability were extracted and analyzed as described above.

To evaluate the extraction recovery, the 10 negative urine samples, used for the validation, were spiked with the peptides at 500 pg/mL and processed together with non-spiked urine samples. The extracts of the non-spiked urine samples were spiked after evaporation and before LC injection. After analysis the obtained peak areas of the two sets of samples were compared.

Matrix effect was evaluated by comparing the peak areas of the urine samples spiked after extraction with a reference solution of peptides at 500 pg/mL, corresponding to a 100% recovery (0% matrix effect).

4 Results and discussion

4.1 UHPLC-HRMS

Analysis of peptides must have very low analytical LODs (in the femtomole range) in order to monitor the misuse of these substances for a realistic period of time. This requires the highest sensitivity allowed by the mass spectrometer. This assumption is based on the pharmacokinetic data currently available from excretion studies[3,13]. Other peptide such as TB-500, are expected to have similar behaviour, although no data on their metabolism are present, as excretion studies are limited by ethical constrains.

The list of small peptides and related metabolites that can be monitored is constantly increasing, as methods are optimized and reference standard material becomes available. In fact, in the nanoLC-HRMS method published by Thomas *et al*. in 2012 eleven peptides (desmopressin, LHRH and 9 GHRPs) were included[5].

The methods described in this work allow for the simultaneous analysis of a total of 31 peptidic substances, including vasopressin analogues, LHRH agonists, GHRPs, TB-500, and AOD9604. All the substances were detected at a concentration of 500 pg/mL or lower.

The use of comprehensive and flexible methods is desirable in the field of doping control. These methods should have a generic sample preparation, untargeted detection and the possibility, in principle, to be extendable to other drugs. In the last few years, the availability of new instrument, particularly HRMS instrument, based on FSMS acquisition, has allowed the development of open screening methods with a virtually unlimited number of compounds, and the possibility to perform retrospective data analysis based on *a posteriori* hypothesis (preventive control analysis)[16,17].

The method was developed on a HRMS instrument, similarly to Thomas *et al*.[5]. 1 µg/mL solutions of the peptides were first injected in order to evaluate their MS behavior. FSMS experiments were first performed in order to evaluate the most abundant ion type. $[M+2H]^{++}$ pseudomolecular ions were generally more abundant than $[M+H]^{+}$ for the peptides, with a few exceptions. GHRP-4, GHRP-5 and most of the metabolites, with a lower molecular weight, showed prevalent monocharged ions (Figure 10.1), whereas the $[M+3H]^{3+}$

was the most abundant for AOD-9604, the biggest among the peptides included in this method (M.W. 1.8 kDa).

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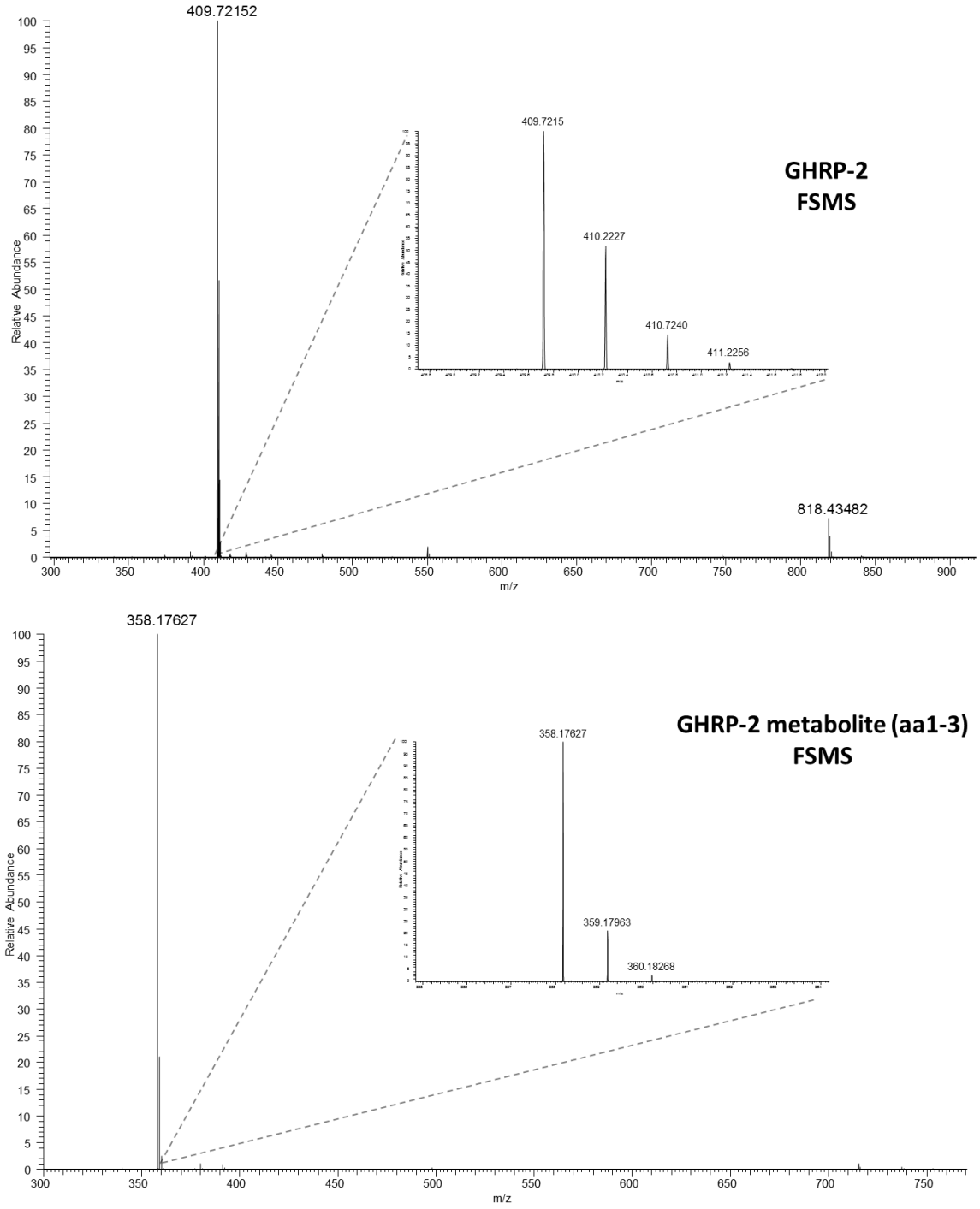

Figure 10.1 Full scan MS spectra of GHRP-2 and GHRP-2 metabolites (AA 1-3).

HRMS data typically show the isotopic pattern of the ions. This is useful in peptide analysis, since it provides additional information by assessing the charge of the ions. This can help the analyst to decide whether to proceed with confirmation of a suspicious sample.

Theoretical *m/z* values of the most abundant species were calculated and then added in the inclusion list. The inclusion list (Table 10.1) contains all the *m/z* values which are monitored during tSIM experiments. The second isotope was selected for GHRP-1, leuprolide and goserelin because of a higher S/N ratio than the monoisotopic peak (*italic* in Table 10.1).

Compared to FS, tSIM yielded lower LODs, but FSMS was also included in the method since it enables retrospective data evaluation for presently unknown peptides or metabolites.

The use of multiplexed (MSX) option was enabled, with the possibility to acquire 10 tSIM experiments in the same scan event, allowing for the acquisition of a higher number of data points. However, the number of data points decreases with increasing the number of the ions in the inclusion list. The use of detection windows (±2 minutes) and a long chromatographic runtime (35 minutes, the same as in the work from Thomas) for a better separation of the species (Figure 10.2), improved reproducibility and accuracy by detecting more data points across chromatographic peaks.

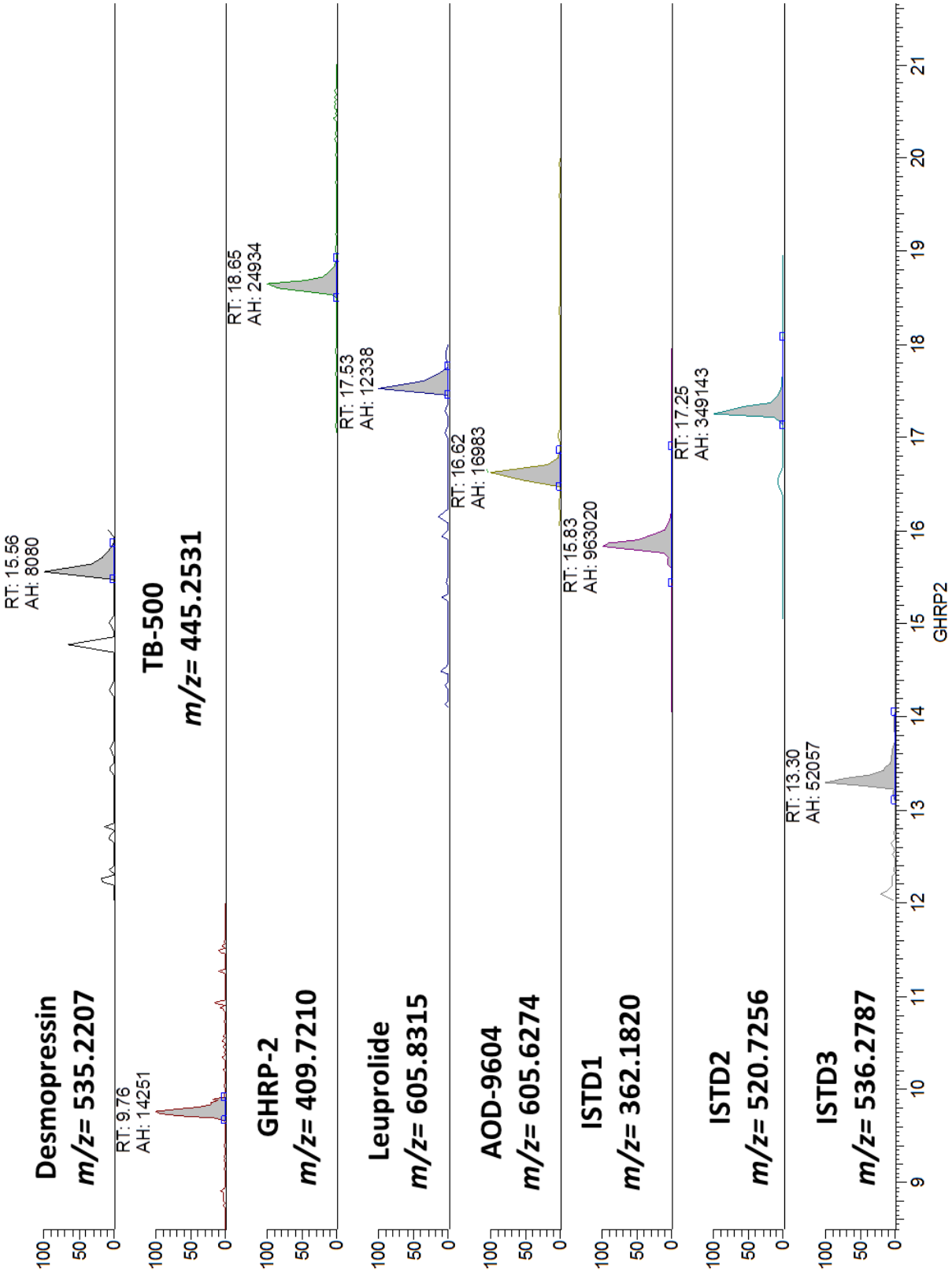

Figure 10.2 Extracted ion chromatogram from representative peptides of different classes detected at their LOD in tSIM mode and the internal standards.

4.2 Sample preparation

As already stated, sample preparation is a critical issue for peptide analysis. Non-specific absorption on surface, variable recovery, loss of analyte during transfer and drying steps can cause poor or irreproducible results if adequate countermeasures, such as using low-bind plasticware and limiting the number of vial transfers, are not taken.

Mechanism of retention/elution of mixed-mode SPE columns is based both on hydrophilic-lipophilic balance and ion exchange. In our previous work on desmopressin, Oasis WCX® were successfully used, whereas Strata X-CW® were used in the method from Thomas *et al.*[5]. Other cartridges, including MM1® and MM2® from InterChrom, Oasis MCX® and Strata X-C® were evaluated, but they showed not adequate selectivity and/or recovery.

The pH of the sample when loaded on the column was an important factor. The pH of urine can range from a low of 4.5-5 to a high of 8. pH values are also important for ion exchange interaction, since it determines whether the analyte or the resin is in the ionized or non-ionized form. Different buffers were therefore tested, including formiate buffer (pH 3.8), acetate buffer (pH 5.2), phosphate buffer (pH 7) and carbonate buffer (pH 9.5). The best results were obtained with acetate buffer. The method uses only 1 mL of urine. Increasing the urine volume resulted in dirtier extracts which affected retention time stability.

4.3 Validation

No minimum required performance level (MRPL) values have been established so far by WADA for small peptides. However, according to the data available in literature for some of the peptides included in the method, a method for small peptides should be developed with the aim to reach sub-ng/mL detection for all the substances.

As shown in Table 10.2, detection was achieved for 26 out of 31 substances at 100 pg/mL or lower. This concentration can be considered a realistic minimum requested performance level (MRPL) value. However, alexamorelin, terlipressin and three short metabolites (TB-500 M1 and M2, and leuprolide M2) could be detected only at 500 pg/mL, due to the presence of interferences or to low recoveries. As expected in a multiclass screening method, recoveries (10.8 to 125.0 %) and matrix effect (-48 to 65.0%) varied significantly.

Table 10.2 Validation data, including diagnostic ion, LOD, recovery and matrix effect for each peptide

Peptide	Elemental composition	Molecular mass	Retention time (min)	Theoretical *m/z*	Charge	LOD tSIM (pg/ml)	Recovery (%)	Matrix effect (%)
Desmopressin	C46H64N14O12S2	1068,4269	15,5	535,2207	++	50,0	95,6	42,4
Lys-vasopressin	C46H65N13O12S2	1055,4317	11,7	528,7230	++	50,0	78,5	37,8
Terlipressin	C52H74N16O15S2	1226,4961	12,8	614,2552	++	500,0	30,7	24,3
TB-500	C38 H69N10O14	889,4995	9,7	445,2531	++	20,0	92,7	38,5
TB-500 M1 (1-2)	C14H27N3O4	301,2001	7,6	302,2074	+	500,0	10,8	46,9
TB-500 M2(1-3)	C20H40N5O5	430,3029	4,0	430,3024	+	500,0	75,2	-49,4
TB-500 M3 (1-5)	C29H54N7O10	660,3932	8,0	330,7002	++	20,0	64,1	-43,3
GHRP-1	C51H62N12O7	954,4860	16,2	*478,7522*	++	100,0	77,0	54,7
GHRP-2	C45H55N9O6	817,4721	18,7	409,7210	++	50,0	84,4	12,0
GHRP-2 M1 (1-3)	C19H23N3O4	357,1682	15,8	358,1761	+	500,0	62,6	10,7
GHRP-4	C34H37N7O4	607,1292	20,2	608,2979	++	50,0	112,9	32,5
GHRP-5	C43H46N8O6	770,3546	21,8	771,3613	++	50,0	133,0	62,3
GHRP-6	C46H56N12O6	872,4445	16,1	437,2296	++	20,0	94,7	58,2
GHRP-6 M1 (2-5)	C34H36N6O5	608.2747	21,0	609,2824	+	20,0	75,2	34,1
GHRP-6 M2 (2-6)	C40H50N9O5	736,3934	17,9	369,1926	++	50,0	78,9	47,0
Alexamorelin	C50H63O7N13	957,4972	15,5	320,1730	+++	500,0	51,0	29,8
Ipamorelin	C38H49N9O5	711,3851	13,5	356,7001	++	20,0	70,0	-13,7
Hexarelin	C47H58N12O6	886,4607	15,3	444,2374	++	100,0	60,4	50,0
Hexarelin M1 (1-3)	C21H26N6O4	426,2003	11,5	427,2081	+	100,0	95,3	27,2
Hexarelin M2 (2-5)	C35H38N6O5	622,2903	21,3	623,2981	+	20,0	65,5	44,4
LHRH	C55H74N16O14	1181,4315	14,6	591,7914	++	20,0	72,4	62,3
Leuprolide	C59H84N16O12	1208,6455	17,4	*605,8312*	++	20,0	87,6	47,6
Leuprolide M1 (5-9)	C34H54N9O5	668,4248	15,8	344,7292	++	50,0	98,8	32,1
Leuprolide M3 1(-3)	C22H25N6O5	452,4703	13,1	453,4780	+	500,0	17,4	43,4
Buserelin	C60H86N16O13	1238,6560	18,2	620,3353	++	100,0	99,8	56,6
Deslorelin	C64H83N17O12	1281,6407	18,1	641,8276	++	20,0	80,4	25,9
Goserelin	C59H84N18O14	1268,6415	17,3	*635,8296*	++	50,0	66,3	37,4
Nafarelin	C66H83N17O13	1321,6356	17,2	661,8251	++	50,0	52,3	23,9
Nafarelin M1 (5-10)	C66H83N17O13	800,4334	18,4	401,2245	++	50,0	125,0	65,0
Triptorelin	C64H82N18O13	1310,6308	17,4	656,3227	++	50,0	64,6	37,7
AOD 9604	C78H123N23O23S2	1812,8588	15,9	605,6274	+++	20,0	61,4	48,7
^{13}C, ^{15}N GHRP-2 M1 (1-3) (ISTD1)	C19H23N3O4	361,1743	15,8	362,1820	+	-	-	-
[deamino Cys1, Val4, D-Arg8] AVP (ISTD2)	C52H75N13O12S2	519,7178	17,2	520,7256	++	-	-	-
LHRH 2-10 (ISTD3)	C52H75N13O12S2	535,2709	13,1	536,2787	++	-	-	-

5 Conclusions and perspectives

A comprehensive UHPLC-HRMS screening method for the detection of 31 small peptide hormones (including 11 metabolites) in human urine has been successfully developed and validated. Detection was achieved at sub-nanogram levels for all the substances. Sample preparation times are limited 2-3 hours (less than 1 hour for SPE, 1-2 hours for evaporation), and 25-30 samples can be analyzed per day.

It must be stated that this method cannot be used for confirmation analysis, as it is based on the detection of a single diagnostic ion, which is not sufficient to fulfill WADA criteria for qualitative identification in a confirmatory analysis[18]. Methods for confirmation of small peptide hormones, based on low resolution MS (triple quadrupole) are in part already available in our laboratory for several compounds (desmopressin, GHRPs and LHRH). As the development of confirmation methods for the rest of the peptides is still ongoing by the time this thesis is being written, this part was not included.

Future studies will be focused on further improvements of method sensitivity, perhaps with the evaluation of the use of nanoLC system, as the LOD values of the method published by Thomas et al.[5] (which, on the other hand, included only 11 peptides from only three different classes) are at least 10-fold more sensitive than the one described here. According to the data already available in literature[1,3-5,13], small peptides generally reach very low urinary concentrations (lower than 100 pg/mL) in a few hours, making detection of their misuse difficult and confirmation even more complicated. This method is supposedly able to detect small peptides and their metabolites up to 1-2 days, which is a limited amount of time in the doping control context. The only exception is represented by desmopressin and its analogues, whose hemodilution effect last for only few hours[8]. For the other small peptides which exhibit mostly anabolic effects, prolonged detection and confirmation remain a big challenge.

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Part VI - Summary and conclusions

Chapter 11

Summary and conclusions

This thesis described the implementation of the analysis of small peptide hormones in the Ghent Doping Control Laboratory. The topic has been tackled with an utmost comprehensive approach. In fact, research has been conducted on all the aspects of the analysis, including 1) investigation of the detectability of new substances (desmopressin), 2) identification of emerging peptide hormones with doping potential from illegal markets, 3) development of alternative *in vitro* models for the study of peptide metabolism and, finally, 4) implementation of comprehensive multi-class, multi-analyte screening and confirmation methods for detection of prohibited small peptides.

1 Detection of desmopressin in plasma and urine

In this first part, detection of desmopressin in human plasma and urine by LC-MS/MS is described. In 2011, desmopressin was added by WADA to the Prohibited List as it can mask blood doping by altering blood parameter used in the framework of the Athlete Biological Passport[1]. Detectability was investigated both in plasma and urine, in order to determine which matrix allowed the longest detection window for the different administration routes (intravenous, oral, intra-nasal).

In **Chapter 3**, the LC-MS/MS method for detection in plasma is described. Detection of the peptide in the biological matrix was achieved by LC-MS/MS (triple quadrupole), after protein precipitation, SPE with WCX cartridges, and HPLC separation.

Detection of desmopressin in urine is described in **Chapter 4**. Same SPE protocol and LC-MS/MS conditions were used. Additionally, a double (acidic/basic) delipidation step of the urine samples was used to obtain a better sample purification.

The methods were both validated and showed good sensitivity (LOD= 50 pg/mL in plasma and 20 pg/mL in urine), with the required specificity and selectivity.

Results of the excretion studies demonstrated that urine is the preferred matrix for the detection of desmopressin misuse in sport as it allows detection of the peptide after oral, intra-nasal or intravenous administration, whereas in plasma detection was possible only

after intravenous administration. Results in urine were also confirmed by the work of Thomas *et al*.[2].

2 Black market of prohibited peptides

In **Chapter 5**, the illegal trade of peptide hormones was discussed. This issue has become particularly relevant, as black markets appear as the main source for purchasing performance-enhancing peptides. Besides being clearly a threat to sport ethics, this phenomenon represents a big concern for public health as well. In fact, many unapproved drugs are marketed despite the risk associated to short and long term toxicity[3]. Furthermore, these products are manufactured without respect for GMP.

In collaboration with Belgian Customs, the Ghent Doping Control Laboratory has analyzed and identified numerous confiscated products containing prohibited peptides/proteins of various molecular masses. Peptides were identified using high-resolution mass spectrometry using both top-down and bottom-up approaches. In particular, three peptides were identified for the first time (**Chapter 6-8**).

In **Chapter 6**, a C-term amidated version of human MGF was identified in two different formulations. The first one was confiscated by the Belgian Customs, whereas the second was purchased from an online shop. Both vials lacked quali-quantitative information. MGF must be considered a potential doping agent, as MGF has a role in repair of muscle damage and other anabolic processes.

In **Chapter 7**, the first identification of a new GHRH analogue with doping potential in a confiscated product was described. The 46 AA presented an unusual sequence, particularly for the presence of a valine residue in position 14. Moreover, the N-terminus was protected by degradation by the addition of a proline. The C-terminus of the peptide was amidated; however, analysis of the products from trypsin digestion revealed an incomplete amidation. This appears to be a clear sign of poor manufacturing quality, together with the absence of any label on the vial.

The characterization of TB-500, described in **Chapter 8**, was related to a case who received also relevant attention from the media, as involved a former cyclist was involved caught up in an investigation after Belgian custom officials intercepted a package containing the product. TB-500 is suspected to be used both for horse and human doping. The active content of TB-500 was identified as the N-term acetylated 17-23 fragment of the 44 AA peptide thymosin β4. After sequence identification by high-resolution mass spectrometry, the peptide was also synthesized to be used as reference standard material for the development of analytical methods. Furthermore, *in vitro* metabolic studies were performed, and three metabolites (Ac-LK, Ac-LKK and Ac-LKKTE) were also synthesized.

The phenomenon of the black markets represents therefore a relevant, alarming aspect in the misuse of peptide hormones. From the analyst point of view, a continuous monitoring of these formulations is essential to identify new potential doping substances toward the implementation of dedicated analytical methods. This should be done in collaboration with Health and Control Authorities which are, in turn, responsible for the promotion and the execution of deterring policies.

3 *In vitro* models for metabolic studies of small peptide hormones

As shown in literature, monitoring peptide metabolites can provide a longer detection window than the parent compound[4]. However, excretion studies on unapproved peptides are strongly limited by ethical constraints. Therefore it was necessary to develop alternative *in vitro* models to perform metabolic studies.

In **Chapter 9**, novel and established *in vitro* models for studying the metabolism of small peptides were evaluated and compared. In particular, the use of human microsomes and S9 fraction for metabolic studies of peptides was described for the first time. So far, the use of these models was limited to “classic” small molecules, but both microsomes and S9 fractions demonstrated to be a valid tool for the identification of potential metabolites, providing

results comparable to previously described models (incubation with fresh serum)[5] in terms of number of metabolites that were identified. The advantages of using microsomes/S9 fraction instead of fresh serum include a simpler sample preparation, fast incubation times and a limited amount of matrix interferences. The use of microsomes from different organs (liver and kidney) showed identical results.

Additionally, deamidation was investigated using incubation with deamidating enzymes (α-chymotrypsin), as neither incubation with microsomes/S9 fraction nor with fresh serum revealed the formation of these metabolites.

All the metabolites that were described in literature as detected *in vivo* were also identified in the *in vitro* models. In conclusion, microsomes, S9 fraction and fresh serum are all useful tools not only for forensic, but also clinical analysts, to detect potential peptidic metabolic markers in biological fluids.

4 Detection of small peptide hormones

Finally, all the studies performed on desmopressin (**Chapter 3 and 4**), black market products (**Chapter 5-8**) and peptide metabolism (**Chapter 9**) found their natural conclusion in the development of a multi-analyte, multi-class UHPLC-HRMS method described in **Chapter 10**. In fact, the final goal of the thesis was the implementation in routine of the analysis of small peptide hormones in the laboratory.

Sample preparation was based on weak cation exchange SPE, also described in other works[6,7]. An open screening method was developed on a UHPLC-HRMS system (Orbitrap® Q-Exactive), similarly in the 2012 work from Thomas *et al*.[6]. Detection was achieved in positive tSIM mode, monitoring one pseudomolecular ion for each peptide. Additionally, a positive FSMS spectrum was constantly acquired to allow for retrospective data evaluation. A total of 31 compounds, including 11 metabolites were included in both methods. Three different internal standards were used. Only 1 mL of urine was used. LODs were in the femtomole (sub-ng/mL) range for all the compounds.

5 General conclusions

This thesis summarizes the effort that has been put by our laboratory in the last years to implement the analysis of small peptide hormones in routine, but also to contribute with research to increase the knowledge on the misuse and the analytical issues of this class of doping agents. Sample pretreatment strategies appear well defined, with the use of SPE. In this area, it is possible to expect improvements in sample throughput capabilities.

On the other hand, there is still room for improvements in the detection capabilities, in order to increase detection and confirmation windows. Both low-resolution (triple quadrupole technology) and high-resolution (Orbitrap® technology) were used in this thesis. In particular, the first was used for the detection of desmopressin in plasma and urine, whereas the multianalyte screening method was developed on the HRMS instrument.

Although a direct comparison was not made in this work, both technologies show advantages and disadvantages in their application to doping control analysis of small peptides.

The measured LODs for desmopressin in urine are similar(25 pg/mL versus 50 pg/mL). However, the method described in **Chapter 4** can be used for confirmation analysis, since two MRM transitions were validated at the method LOD. The HRMS method (**Chapter 10**) uses only one transition, based on the tSIM detection of the pseudomolecolar ion and therefore it can only be used for screening. On the other hand, this acquisition mode provides accurate mass data and information on the isotopic cluster of the ion. At the same time, full scan acquisition allows for retrospective analysis and fast implementation of new peptides. For this reason, it represents a valid choice for screening analysis.

MSMS experiments on the HRMS instruments (targeted MSMS and data-dependent MSMS) were also performed, but the method did not show adequate LOD (data not shown). So far, there is only one work that describes sensitive detection of a small peptide (desmopressin) using HRMS[2]. Further improvements are still needed in order to implement routine use of HRMS for confirmation analysis of small peptides.

Future research on small peptides will be oriented mainly in the optimization of methods. Sensitivity remains the top priority for this analysis, due to the short detection window for these substances. Improvements in this direction can be obtained taking advantage of technologic advances (new LC-MS systems or better purification strategies) and characterization of additional long-term metabolites. Moreover, there is room for improving time- and cost-effectiveness of methods.

The first method describing detection of a single, small peptide (LHRH) has been published in 2008[8]. Since then, significant progresses have been made thanks to the research performed by WADA laboratories. Thanks to all this work, protocols for the development of detection methods, metabolic studies and identification of new peptidic doping agents have been established.

At the same time, the research has contributed to increase WADA awareness on the topic. Therefore, it is expected that a work of harmonization will be done, providing adequate qualitative (dedicated identification criteria) and quantitative (MRPL) parameters.

In conclusion, it is legitimate to predict that the majority of the laboratories will implement analysis of small peptides in the next few years, similarly to what has been done for other classes in the past, and thus marking another step forward in the fight against doping.

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Chapter 12

Samenvatting and conclusies

Het onderwerp ‘kleine peptide-hormonen’ werd in al z’n facetten onderzocht. In een eerste deel van de thesis wordt de analyse van een klinisch relevante substantie (desmopressine) onderzocht. Het tweede deel spitst zich toe op de identificatie van illegale preparaten die peptide-hormonen bevatten. In een derde deel wordt een *in vitro*-model besproken om metabolisatie van peptide-hormonen te onderzoeken. In het vierde, en laatste, deel wordt de ontwikkeling en validatie beschreven van een methode om peptide-hormonen routinematig op te sporen in urine.

1 Detectie van desmopressine in plasma en urine

In dit deel van de thesis werd de detectie van desmopressine in humaan plasma en urine met behulp van LC-MS/MS beschreven. Desmopressine is een klinisch relevant peptide en wordt gebruikt in de behandeling van o.a. *enuresis nocturna*. In 2011, werd desmopressine toegevoegd aan de verboden lijst van WADA omdat desmopressine het opsporen van bloeddoping kan bemoeilijken. Dit is belangrijk in het kader van het biologisch paspoort[1]. Zowel in plasma als in urine werd de detecteerbaarheid onderzocht. Dit had tot doel om te bepalen welke matrix het meest geschikt is om de verschillende administratie-routes te detecteren.

In hoofdstuk 3 wordt de detectie in plasma beschreven. Om de plamsa-stalen op te zuiveren werd er eerst een proteïne-precipitatie uitgevoerd, gevolgd door een vaste-fase-extractie met zwakke kationen-uitwisselaar (type WCX). Finale detectie van het peptide werd bekomen met behulp van LC-MS/MS.

De detectie van desmopressine in urine wordt beschreven in hoofdstuk 4. Dezelfde vaste fase extractie-strategie en detectie methode werd gevolgd als bij de plasma-experimenten. Bijkomend werd een dubbele (zuur/base) delipidatie stap ingevoerd om de urinestalen extra op te zuiveren.

Zowel de detectie in urine als in plasma werd gevalideerd. In plasma werd een detectiegrens (LOD) bekomen van 50 pg/mL. In urine kon detectie van desmopressine gevalideerd worden

tot op 20 pg/mL. Er werd ook voldaan aan de vooropgestelde specificiteit en selectiviteit. De resultaten zijn in overeenstemming met het werk van Thomas *et al.*[2].

De excretiestudies tonen aan dat urine de preferentiële matrix is om sport-gerelateerd desmopressine misbruik op te sporen. Bij alle onderzochte administratieroutes (oraal, intraveneus en intranasaal) kon desmopressine gedetecteerd worden. In plasma kon desmopressine enkel gedetecteerd worden na intraveneuze toedieningswegen.

2 Illegale handel van kleine peptide-hormonen.

In hoofdstuk 5 wordt de illegale handel van peptide-hormonen beschreven. De illegale handel van peptide-hormonen is in een hogere versnelling gekomen door de beschikbaarheid van het internet. Dit medium laat toe om volstrekt anoniem, illegale producten aan te kopen. Niet alleen vervalst het gebruik van deze peptide-hormonen sportprestaties, ze vormen ook een bedreiging voor de volksgezondheid aangezien de toxiciteit van deze substanties onbekend is[3]. Bovendien worden deze substanties vervaardigd in niet traceerbare industrieën, waardoor enige vorm van kwaliteitsborging (GMP) ontbreekt. DoCoLab heeft in samenwerking met de douane, verschillende illegale preparaten onderzocht die mogelijks verboden peptide-hormonen bevatten. Dit onderzoek leidde tot de identificatie van 3 nieuwe peptide-hormonen. De peptides werden geïdentificeerd met hogeresolutiemassaspectrometrie met zowel “top-down” als “bottom-up” approach (hoofdstuk 6-8).

In hoofdstuk 6 wordt de identificatie van een geamideerde (C-terminus) versie van het humane MGF besproken. MGF moet beschouwd worden als een dopeermiddel aangezien MGF een rol speelt bij het herstellen van spierweefsel en betrokken is bij verschillende anabole processen in het menselijke lichaam.

Dit peptide werd teruggevonden in twee verschillende preparaten. Het eerste preparaat werd in beslag genomen door de Belgische douane en het tweede preparaat werd aangekocht via internet. Voor beide preparaten ontbrak elke vorm van kwaliteitsgarantie.

In hoodstuk 7 wordt de detectie en identificatie van een GHRH-analoog besproken. Dit peptide-hormoon werd ook teruggevonden in een geconfisqueerd product.

Het peptide-hormoon bestaat uit 46 aminozuren, en heeft een abnormale sequentie, aangezien op positie 14 een valine-aminozuur is ingebouwd. Bovendien is de N-terminus beschermd tegen degradatie door toevoeging van het aminozuur proline. Bovendien was de C-terminus van het peptide geamideerd. Naast het geamideerde peptide werd er in het geconfisqueerd product ook niet geamideerd-peptide gedetecteerd. Dit wijst op een incomplete amidatie en is nogmaals een bewijs voor de ongecontroleerde productie en de afwezigheid van enige kwaliteitsborging.

De karakterisatie van TB-500 is beschreven in hoofdstuk 8 en kreeg heel wat media aandacht omdat een voormalige wielrenner dit preparaat aankocht via het internet.

Van TB-500 wordt verondersteld dat het gebruikt wordt voor zowel humane als paardendoping. De actieve component van TB-500 werd geïdentificeerd als het N-terminaal geacetyleerde fragment 17-23 van het, uit 44 aminozuren bestaande, peptide-hormoon thymosine β4. Na identificatie van de aminozuur-sequentie mbv hoge resolutie massaspectrometrie, werd het peptide ook gesynthetiseerd. Het gesynthetiseerde product kan gebruikt worden als referentie standaard om analytische methodes, voor de detectie van TB-500, op punt te stellen. Bijkomend werden, in het kader van een WADA-project, 3 metabolieten gesynthetiseerd (Ac-LK, Ac-LKK and Ac-LKKTE). Het bestaan van deze metabolieten werd mbv een *in vitro* model vastgesteld.

Er kan besloten worden dat het fenomeen van de “zwarte-internet- markt” een relevant en alarmerend probleem is. Daarom is het belangrijk dat laboratoria verantwoordelijk voor volksgezondheid deze nieuwe preparaten, die te koop worden aangeboden, controleert op de aanwezigheid van nieuwe illegale peptides. Dit onderzoek kan enkel verlopen in nauwe samenwerking met douane en andere federale instellingen die de producten in beslag kunnen nemen.

3 *In vitro* modellen voor metabole studies van kleine peptide-hormonen

Peptide-hormonen worden snel gemetaboliseerd. Daarom kan het monitoren van metabolieten het detectievenster verlengen[4]. Om de metabolisatie te onderzoeken dient het peptide-hormoon toegediend te worden aan gezonde vrijwilligers. Aangezien de toxiciteit van deze illegale peptide-hormonen niet gekend is, kunnen ze ook niet toegediend worden aan vrijwilligers om ethische redenen.

In hoofdstuk 9 worden alternatieve modellen om de metabolisatie te onderzoeken, voorgesteld en vergeleken. In het bijzonder het gebruik van humane microsomen en S9-fracties wordt voor de eerste maal beschreven in het kader van peptide-metabolisatie. Tot op heden werd het gebruik van microsomen en S9-fracties enkel beschreven om metabolisatie van kleine molecules te bestuderen. In deze thesis werd aangetoond dat microsomen en S9 fracties geschikt zijn om potentiële peptide-metabolieten te identificeren. De resultaten zijn gelijkaardig aan reeds vroeger beschreven modellen, o.a. incubatie met vers serum[5]. Het voordeel van microsomen en S9-fracties ten opzichte van vers serum is de eenvoudige staalvoorbereiding, korte incubatietijden en weinig interferentie. Er werd geen significant verschil in metaboliet-vorming waargenomen tussen nier en lever-microsomen.

Deamidatie werd met behulp van het deamiderende enzyme (α-chymotrypsin) onderzocht, omdat noch microsoom, S9 fracties , noch serum in staat zijn om peptides te deamideren.

4 Detectie van kleine peptide-hormonen

De verzamelde kennis beschreven in de voorgaande hoofdstukken over desmopressin (hoofdstuk 3 en 4), illegale preparaten (Hoofdstuk 5-8) en peptide-metabolisme (Hoofdstuk 9) leidde tot de ontwikkeling van een UHPLC-HRMS detectiemethode om kleine peptide-

hormonen in urine te detecteren in het kader van dopinganalyse. Dit wordt beschreven in hoofdstuk 10.

De staalvoorbereiding bestond uit een zwakke kationen-uitwisseling vaste fase extractie[6,7]. Slechts 1 mL urine was nodig voor de extractie. De detectie werd uitgevoerd mbv een UHPLC-HRMS systeem (Orbitrap® Q-Exactive) in overeenstemming met het werk beschreven door Thomas *et al.*[6] in 2012. Alle peptides werden gedetecteerd in positieve ionisatie modus (SIM). Voor elk peptide werd 1 geprotoneerd moleculair ion gemonitord. In totaal werden 31 substanties (waarvan 11 metabolieten) aan de methode toegevoegd. Drie verschillende inwendige standaarden werden gebruikt. De validatie toonde aan dat alle substanties tussen 20 en 500 pg/mL kunnen gedetecteerd worden.

5 Algemeen besluit

Deze thesis beschrijft de implementatie van de detectie van peptide-hormonen in het dopingcontrolelaboratorium van de Universiteit Gent.

Dit onderzoek omvatte niet enkel de implementatie van het routinematig opsporen van peptide-hormonen maar ook het karakteriseren en identificieren van nieuwe peptide-hormonen.

DoCoLab zal de in het kader van deze thesis ontwikkelde methode verder optimaliseren.

Aangezien peptide-hormonen doorgaans in lage dosissen worden toegediend en ze snel gemetaboliseerd worden is “gevoeligheidsverbetering van de detectiemethodes” een belangrijk onderzoekspunt.

Gevoeligere methodes kunnen vooral bekomen worden door technologische vooruitgang die ons betere massaspectrometers levert. Daarnaast kan een selectievere staalvoorbereiding of de identificatie van metabolieten die lang in het lichaam aanwezig blijven ook een substantiële verbetering in de detectie toelaten. Deze potentiële verbeteringen zullen ook de economische rentabiliteit van deze detectiemethodes ten goede komen.

Sinds de publicatie van de eerste detectie-methode, die de detectie van slechts een klein peptide bescheef (LHRH), in 2008[8] , werd er heel wat vooruitgang geboekt in de detectie van de peptide-hormonen. Deze vooruitgang is te danken aan het wetenschappelijk onderzoek in de verschillende WADA-laboratoria. Door dit onderzoek beschikken dopinglaboratoria momenteel over algemene procedures voor de ontwikkeling van detectiemethodes, uitvoeren van metabole studies en de identificatie van nieuwe peptide-hormonen. Deze algemene procedures laten toe dat alle 32 geaccrediteerde laboratoria in de volgende jaren peptide-detectie-methodes implementeren. Om de implementatie van deze methodes in alle laboratoria op dezelfde manier te laten verlopen is er nood aan harmonisatie. In het bijzonder ontbreken er voor verschillende peptides referentie-standaarden en zijn er nog geen minimum required reporting levels (MRPL). In deze harmonisatie dient WADA een voortrekkersrol te nemen.

6 Referenties

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Curriculum Vitae

1 Personal information

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2 Education and work experience

2010-2014

DoCoLab - Ghent University; Ghent, Belgium

PhD candidate

Research topics:

Development of analytical methods for the detection of small peptide doping agents by LC-MS.

Identification of unknown peptide drugs in black market formulations.

Promotor: Prof. Dr. Ir. Peter Van Eenoo

Co-promotor: Dr. Koen Deventer

2009-2010

Laboratorio Antidoping FMSI; Rome, Italy

Postgraduate researcher

Reseach topics:

Evaluate the potential masking effect of liposomes on the detection of androgenic anabolic steroids by GC-MS, LC-MS and flow cytometry.

2003-2009

Master's degree in Pharmaceutical Chemistry and Technology, "Sapienza" University of Rome, Rome, Italy

Thesis title: "Evaluation of potential masking agent of substances not included in the Antidoping lists. Study of liposomes-steroids interaction"

1998-2003

Liceo Classico A. Mancinelli, Velletri, Roma

3 Scientific Contributions

3.1 Publications

1. **S. Esposito**, K. Deventer, P. Van Eenoo. Characterization and identification of a C-terminal amidated mechano-growth factor (MGF) analogue in black market products. Rapid Comm. Mass Spectr. 2012, 26, 686.

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2. F. Botrè, **S. Esposito**, X. de la Torre. How We Risk: Liposomes and Steroids. Proceedings of the 29th Cologne Workshop on Dope Analysis (2011).

3. **S. Esposito**, K. Deventer, P. Van Eenoo. Doping control analysis of desmopressin in human plasma and urine by HPLC-ESI-MS/MS. Proceedings of the 30th Cologne Workshop on Dope Analysis (2012).

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3.3 Oral presentations

1. **S. Esposito**, K Deventer, J Goeman, J Van der Eycken, P Van Eenoo. TB-500: from horseracing to human doping. 30th Cologne Workshop on Dope Analysis 2012, Cologne, Germany.

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3.4 Posters

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3.4 Books

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